REMARKS

Reconsideration is requested.

Claims 1-42, 44, 48-51, 59, 60, 62, 67 and 79 have been canceled, without prejudice. Claims 43, 45-47, 52-58, 61, 63-66 and 68-78 are pending.

Support for revised claims 68 and 77 can be found throughout the specification. Specifically, for example, the applicants believe the revisions are supported by page 11, line 25 to page 12, line 6 of the specification. No new matter has been added.

The allowance of claims 43, 45-47, 53-58, 61 and 64-66 is acknowledged, with appreciation. See page 1 of the Office Action dated April 18, 2007.

A further copy of the previously-listed documents is attached herewith. The PTO fee for consideration of the references is also attached. The Examiner is requested to return a completely initialed copy of the previously-filed PTO 1449 Form listing the references, pursuant to MPEP § 609, as acknowledgement of the consideration of the references.

The Rule 75 objections to claims 51 and 62 are moot in view of the above. The applicants believe Rule 75 objections of claim 52 and 61 are obviated by the above amendments. Withdrawal of the objections is requested.

The Section 112, second paragraph, rejection of claims 48-50 is moot in view of the above.

The Section 112, second paragraph, rejection of claims 68-78 is obviated by the above amendments. Specifically, claim 68 recites that a pre-adipocyte cell according to claim 61 is prepared by introducing into a pre-adipocyte cell a recombinant nucleic acid comprising a region coding a REV ERB ALPHA receptor comprising SEQ ID NO:4 and

one or more transcriptional regulatory regions. Claims 76 to 78 further define the transcriptional regulatory regions. Claim 78 defines a transcriptional regulatory region that comprises sequence SEQ ID NO:1 or a fragment thereof comprising at least SEQ ID NO:2. The claims are submitted to be definite and withdrawal of the Section 112, second paragraph, rejection of claims 68-78 is requested.

The Section 112, first paragraph "enablement", rejection of claim 51 is moot in view of the above.

The Section 112, first paragraph "enablement", rejection of claim 52 is believed to be obviated by the above amendments, based on the Examiner's comments on page 6 of the Office Action dated April 18, 2007. Reconsideration and withdrawal of the Section 112, first paragraph "enablement", rejection of claim 52 is requested.

The Section 112, first paragraph "written description", rejection of claims 44, 48 and 49, is moot in view of the above.

The Section 112, first paragraph "written description", rejection of claim 51, is moot in view of the above.

The claims are submitted to be in condition for allowance and a Notice to that effect is requested. The Examiner is requested to contact the undersigned, preferably by telephone, in the event anything further is required to place the application in condition for allowance.

Respectfully submitted,

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(54) Title: USE OF REV–ERB FAMILY OF RECEPTORS IN SCREENING

(57) Abstract

The present invention relates to the use of receptors of the Rev–erb family to screen substances which are useful in the treatment of lipid metabolism dysfunctions associated with apolipoprotein C–III. The invention relates more particularly to screening methods for selecting substances which are useful for the treatment of these dysfunctions. Lastly, the invention relates to the use of the substances thus identified for the preparation of therapeutic compositions which are useful for the treatment of lipid metabolism dysfunctions associated with apolipoprotein C–III. The subject of the present invention is also the use of screening tests for the characterization, justification and claim of the mechanism of action of substances possessing anti–atherosclerotic properties using the Rev–erb receptors and/or the response elements thereof, as well as their effect on apo C–III.

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USE OF REV-ERB FAMILY OF RECEPTORS IN SCREENING

The present invention relates to the use of receptors of the Rev-erb family to screen substances which are useful in the treatment of lipid metabolism dysfunctions associated in particular with apolipoprotein C-III. The invention relates more particularly to screening methods for selecting substances which are useful for the treatment of these dysfunctions. Lastly, the invention relates to the use of the substances thus identified for the preparation of therapeutic compositions which are useful for the treatment of lipid metabolism dysfunctions associated with apolipoprotein C-III, such as, for example, atherosclerosis. The subject of the present invention is also the use of screening tests for the characterization, justification and claim of the mechanism of action of substances possessing anti-atherosclerotic properties using the Rev-erb receptors and/or the response elements thereof, as well as their effect on apo C-III.

Apolipoprotein C-III, referred to hereinbelow as apo C-III, is a glycoprotein of 79 amino acids synthesized in the liver and, to a lesser extent, in the intestine. It plays a major role in the metabolism of the plasma triglycerides. In point of fact, the plasma concentrations of apo C-III are positively correlated with the plasma levels of triglycerides, both in the normal population and in hypertriglyceridaemic patients (1-4). Furthermore, the relative distribution of apo C-III with respect to the other classes of lipoproteins appears to be large: an increase in the concentration of apo C-III in particles which contain apo B (apo C-III-LpB) is associated with an increased risk of cardiac or coronary diseases (5). Several lines of evidence link apo C-III with the catabolism of plasma triglycerides.

A deficiency in apo C-III is reflected by an increase in the catabolism of the very low density particles (VLDL), whereas an increase in the synthesis of apo C-III appears in hypertriglyceridaemic patients (6, 7).

Furthermore, genetic studies have revealed the relationship existing between certain polymorphisms of the apo C-III gene and high concentrations of triglycerides and of apo C-III in the plasma (8, 9).

Lastly, the overexpression of human apo C-III in transgenic animals was reflected by the development of hypertriglyceridaemia, whereas the deletion of the endogenous apo C-III gene by homologous recombination in mice led to a decrease in the plasma concentration of apo C-III and to protection of the animal against postprandial hypertriglyceridaemia (10, 11).

The results of studies carried out *in vivo* and *in vitro* indicate that apo C-III acts mainly by retarding the catabolism of triglyceride-rich particles, either by inhibiting their binding to the surface of the endothelium and the subsequent lipolysis with the lipase lipoprotein, or by interfering with the clearance of the residual particles (remnants) which is ensured by the apo E receptor (12-16).

Lastly, the importance of apo C-III in the metabolism of lipoproteins is also suggested by the observation of several characteristics of combined familial hyperlipidaemia (large amounts of VLDL and LDL associated with early cardiac and coronary diseases) in the descendants of crosses between mice whose low density particle (LDL) receptor gene has been removed by homologous recombination and mice which overexpress the human apo C-III gene (17).

The Rev-erb nuclear receptors form a subfamily of orphan nuclear receptors encoded by at least three different genes, Rev-erbα(ear1), Rev-erbβ(BD73,ear4,RVR) and HZF-2(Rev-erbγ) (18-25), the natural ligands of which are currently unknown. The mRNA coding for the Rev-erbα nuclear receptor is expressed in many tissues,

particularly in muscle, brown adipose tissue and the brain (26). Expression of the Rev-erbα gene is induced during adipocyte (26) and myocyte (53) differentiation and in the liver in response to a chronic treatment with fibrates (59). This expression also appears to follow a circadian rhythm (55). The two genes Rev-erbβ and Rev-erbγ are expressed in particular in the brain (22, 25). Rev-erbα and Rev-erbβ can bind as monomers to a response element consisting of a half-site PuGGTCA preceded by an A/T-rich region of 5 base pairs (A/T-A-A/T-N-T-A/G-G-G-T-C-A) (28, 21). A dimeric binding of Rev-erbα on a direct repetition of two AGGTCA half-sites separated by two base pairs and preceded by an A/T-rich region has also been described *in vitro* (29). The crystallographic structure of the complex formed from the DNA binding domain of Rev-erbα with the direct repetition of the two AGGTCA half-sites has been described (54). In contrast with what had initially been described (28), it appears that the nuclear receptors of the Rev-erb subfamily repress the transcription (29, 20). Several physiological targets of Rev-erbα have been identified to date: the oncogene N-myc (30), the rat apo A-I gene (27), the human hRev-erbα nuclear receptor itself (31) and the transcription factors myoD and myogenin (53).

The studies by the Inventors have shown that the Rev-erb receptors are negative regulators of transcription of the apo C-III gene. These receptors are thus capable of repressing the transcription of the apo C-III gene which is associated with the development of hypertriglyceridaemia and hyperlipidaemia.

The present invention thus relates to the use of Rev-erb receptors and/or one of the response elements of these receptors or a functional equivalent thereof to screen substances which are useful in the treatment of lipid metabolism dysfunctions. In addition, the present invention relates to the use of a screening process for the characterization, justification and claim of the mechanism of action of

substances possessing anti-atherosclerotic properties using the Rev-erb receptors and/or the response elements thereof, as well as to their effect on apo C-III.

For the purposes of the present invention, the term "Rev-erb receptor" denotes all the α, β and γ isoforms of the Rev-erb family.

The expression "functional equivalent of Rev-erb" means any protein possessing both:

- a ligand binding site having a selectivity which is comparable to that of Rev-erb for a given ligand thereof,

and

- a DNA binding site which recognizes the same response element as Rev-erb or a response element which has a similar nucleic acid sequence.

The expression "functional equivalent of Rev-erb" also means a chimeric protein which has:

- a ligand binding site having a selectivity which is comparable to that of Rev-erb for a given ligand thereof,

and

- a DNA binding site which recognizes a response element of a reporter gene, or a protein domain which allows the ready purification of the chimera and its specific binding to defined matrices such as, for example, maltose binding protein (MBP) or glutathione-S-transferase (GST). The latter type of chimera has often been used (53). It has the advantage of allowing purification of the protein in one step by means of an affinity column or of specifically separating out this protein by means of simple procedures which are well known to those skilled in the art (coupling to beads or resins, elution with maltose or glutathione, etc.).

The expression "functional equivalent of the Rev-erb receptor response element" means any nucleic acid sequence onto which the Rev-erb receptor can bind

and more particularly a sequence derived from the Rev-erb receptor response element.

The hRev-erbα receptor, the hRev-erbα messenger RNA and the hRev-erbα receptor response element are more particularly preferred in the implementation of the invention.

A subject of the present invention is thus a first type of process for screening substances which are useful in the treatment of lipid metabolism dysfunctions, which consists in placing the test substance in contact with a receptor of the Rev-erb family and/or a Rev-erb receptor response element, and/or a nuclear factor capable of functionally coupling Rev-erb to the RNA-polymerase complex, or a functional equivalent thereof, and then in measuring by any appropriate means:

- the binding of the said substance to the Rev-erb receptor and/or its functional equivalent or the binding of the complex formed from the said substance and the Rev-erb receptor to its response element and/or to a nuclear factor capable of functionally coupling Rev-erb to the RNA-polymerase complex,

and/or

- the modulation of the transcriptional activity of a gene placed under the control of a promoter comprising the said response element.

Measurement of the binding of the substance to the Rev-erb receptor and/or to its functional equivalent or the binding of the complex formed from the said substance and the Rev-erb receptor to its response element can be performed by any direct or indirect method known to those skilled in the art, such as methods using a reporter gene, binding tests, etc.

Similarly, measurement of the modulation of the transcriptional activity of a gene placed under the control of a promoter comprising the Rev-erb response element can be performed by any direct or indirect method known to those skilled in the art.

In order to specify the usefulness of the test substance in the treatment of lipid metabolism dysfunctions, the process of the invention comprises an additional step directed towards determining, by any appropriate means, the effect of the said substance on the expression of apo C-III. The determination of the effect of the test substance on the expression of apo C-III can be performed by any direct or indirect method known to those skilled in the art, such as a transfection or an mRNA analysis *in vitro* and on models *in vitro* and *in vivo*.

A first example of a screening process according to the present invention comprises the following steps:

a) a host cell is transfected with a DNA fragment coding for a Rev-erb receptor or a functional equivalent thereof,

b) the host from step (a) is cotransfected with a construct comprising a response element of the said Rev-erb receptor and at least one reporter gene,

c) the expression of the reporter gene in the presence of the test substance is measured by any appropriate means.

The response element used in step (b) may consist, for example, of the proximal fragment of the apo C-III promoter.

Any reporter gene which allows measurement of the activity of nuclear receptors on the sequence comprising their response element can be used in the screening process according to the invention. Among these, mention may be made, for example, of the chloramphenicol acetyltransferase (CAT) gene, the luciferase gene from luciole (Luc) or from Renilla (Ren), the secreted alkaline phosphatase (SAP) gene or the β-galactosidase (β-Gal) gene. The activity of the proteins encoded by these genes can also be easily measured by conventional methods and makes it possible to know the effect of the nuclear receptors on the

expression of the genes by measuring the amount of proteins produced or their enzymatic activity.

The action of the Rev-erb receptors, and more particularly of the hRev-erbα receptor on the apo C-III gene reported by the Inventors makes it possible, of course, to use the Apo C-III gene as a reporter gene in the constructs of the invention and the screening processes using them.

In the screening process of the invention, the term "host cell" means any cell type adapted to the expression of the above genes, such as, in particular, mammalian cells, bacteria or yeasts, or alternatively insect cells. Needless to say, the vectors used are adapted to the type of cell transfected; mention may be made of plasmids, viruses or artificial chromosomes.

Another example of this first type of screening process according to the invention comprises the following steps:

a) a plasmid is created which comprises several copies of a response element recognized by Rev-erb, such as, for example, a site RevDR2 of the Rev-erbα promoter (31), the consensus site described by M. Lazar (28, 29), or the Rev-erb response element(s) identified in the apo C-III promoter. These copies of the response element are cloned upstream of a heterologous strong promoter such as the thymidine kinase promoter of the herpes simplex virus, or a homologous strong promoter such as the apo C-III promoter. This promoter is itself arranged so as to control the expression of a reporter gene such as luciferase, CAT, alkaline phosphatase or β-galactosidase,

b) the construct from step (a) is transfected into cells which express Rev-erb naturally or artificially, i.e. after transient cotransfection of an expression vector or creation of a stable line which expresses Rev-erb, and

c) the host from step (c) is incubated in the presence of the test substance,

d) the activity of the reporter gene is measured by any appropriate means.

The revDR2 sites are Rev-erb response elements onto which the receptor binds as a dimer to modulate the transcriptional activity of the gene placed downstream. These sites can be used to sensitize a heterologous promoter to Rev-erb.

An additional example of this first type of process comprises the following steps:

a) a plasmid is created which comprises several copies of a response element recognized by Rev-erb, which are cloned upstream of a strong promoter which controls the expression of a suicide selection gene such as, for example, the activator of a toxic prodrug such as herpesvirus thymidine kinase (48),

b) the construct from step (a) is transfected into a host cell,

c) the host from step (b) is cotransfected using a vector which expresses Rev-erb, and

d) the host from step (c) is incubated in the presence of the test substance,

e) the cell survival in the presence of the toxic prodrug is measured by any appropriate means.

The toxic prodrug may be, for example, ganciclovir.

Yet another example of this first type of process comprises the following steps:

a) a plasmid is created which comprises several copies of a response element recognized by the yeast nuclear factor Gal4, which are cloned upstream of a strong promoter, such as the thymidine kinase promoter of the herpes simplex virus, which controls the activity of a reporter gene such as luciferase, CAT, alkaline phosphatase, β-galactosidase, growth hormones, etc.,

b) the plasmid of a chimera is created which comprises the DNA binding domain of Gal4 (49) and the DEF domains of Rev-erb which are the Rev-erb domains to which the ligands bind,

c) the plasmids obtained in steps (a) and (b) are cotransfected into a host cell, and

d) the host from step (c) is incubated in the presence of the test substance,

e) the activity of the reporter gene is measured by any appropriate means.

The DEF domains of the nuclear receptors diverge between various members of this family. They comprise sequences involved in transactivation of the transcription and binding of the ligands and cofactors. The DEF domains of Rev-erb are combined with the Gal4 fragment which contains the first 147 amino acids of Gal4 to create a chimera Gal4-Rev-erbDEF which binds to the Gal4 response element and whose transcriptional activity depends on the Rev-erb ligands and/or cofactors (29).

The basal activity of the chimera can be increased by inserting a DNA fragment which codes for all or part of the protein VP16 (50).

The first type of screening process can also be implemented in the following way

a) a plasmid is created which comprises several copies of a response element recognized by the yeast nuclear factor Gal4, which are cloned upstream of a strong promoter which controls the expression of a suicide selection gene, as explained above,

b) a chimera is created which comprises the DNA binding domain of Gal4 and the DEF domains of Rev-erb,

c) the plasmids obtained in steps (a) and (b) are cotransfected into a host cell, and

d) the host from step (c) is incubated in the presence of the test substance,

e) the cell survival in the presence of the toxic prodrug is measured by any appropriate means.

An additional example of this first type of screening process consists of the quantitative evaluation of the effects of the test compounds in systems of "double hybrid" type in yeasts or other cells which comprise the Rev-erb fragments which interact with

cofactors and the corresponding fragments of the cofactors (e.g.: RIP13a, RIP13d1 (51), N-COR (52) or optionally SMRT and P300/CBP) which couple Rev-erb to the transcriptional machinery and in particular to the RNA-polymerase complex.

Another example of the first type of screening method according to the invention consists in quantitatively evaluating the effects of the test compounds on the *in vitro* capacity for interaction between the entire hRev-erbα protein or some of its fragments and cofactors or some of their fragments by any technique known in the prior art (for example by the CARLA approach developed for PPAR ligand screening (45), a method by measurement of the resonance fluorescence energy transfer).

A final example of the first type of screening process according to the invention consists in transforming a host cell as defined above, with a construct bearing a gene coding for the Rev-erb receptor or a functional equivalent thereof and/or a Rev-erb receptor response element, and then in using the said host cells or extracts thereof in "binding" tests based on the competitive displacement between a cold ligand and a labelled ligand.

A subject of the present invention is also a second type of process for screening substances which are useful in the treatment of lipid metabolism dysfunctions, which consists in determining the effect of the test substance on modulation of the expression of Rev-erb.

One example of a screening process based on measuring the modulation of the expression of Rev-erb consists in directly evaluating the effect of compounds on the cell accumulation of mRNA coding for Rev-erb by *in situ* hybridization (Amersham technique), RPA, quantitative or semi-quantitative RT-PCR, dot blotting or Northern blotting.

A second example of determination of the modulation of the expression of Rev-erb consists in

measuring the effect of the test substance on the cell expression of the Rev-erb protein by immunocytochemistry, ELISA or Western blotting.

An additional example of this second type of process consists in indirectly evaluating the activity of the Rev-erb gene promoter. This process comprises the following steps:

a) a plasmid is created which comprises the Rev-erb gene promoter (31) cloned upstream of a reporter gene such as a luciferase, CAT, alkaline phosphatase, β-galactosidase, growth hormone, etc. gene or a selection gene such as a gene for resistance to an antibiotic or to a conversion enzyme of a non-metabolizable precursor,

b) a host cell is transfected,

c) the test substance is introduced,

d) the activity of the reporter gene or the cell survival is measured by any appropriate means.

The Rev-erb promoter controls the expression of the Rev-erb gene and in particular contains a Rev-erb response element responsible for self-inhibition of the transcription of the gene. Constructs comprising fragments of this promoter are available to characterize the factors involved in modulation of the expression of this gene.

An additional example of a process for measuring the modulation of Rev-erb expression consists in measuring the activity of the endogenous promoter of the Rev-erb gene. This process comprises the following steps:

a) a plasmid is created which comprises several copies of a response element recognized by Rev-erb, which are cloned upstream of a strong promoter which controls the expression of a suicide selection gene such as an activator of a prodrug such as the herpesvirus thymidine kinase, or a reporter gene,

b) the construct obtained in step (a) is transfected into a host cell,

c) a stable cell line which expresses this construct and which expresses hRev-erbα is established, and

d) the host from step (b) or (c) is incubated in the presence of the test substance,

e) the cell survival in the presence of the toxic prodrug or the activity of the reporter gene is measured by any appropriate means.

A subject of the present invention is also substances selected by a screening method according to the present invention, as well as the use of these substances for the preparation of a composition, in particular a pharmaceutical composition, which represses the expression of apo C-III and is thus intended for the treatment of lipid metabolism dysfunctions in man or animals. Compounds possessing such properties are selected on the basis of their capacity to repress the expression of apo C-III, and can be ligands of Rev-erb or of Rev-erb analogues, the properties of which are demonstrated either directly from the level of expression of the apo C-III, or by means of the expression of a reporter gene, or alternatively by their capacity to form a complex with the Rev-erb receptor.

The invention thus relates more generally to the use of a substance which is capable of modulating the expression of apo C-III for the preparation of a composition, in particular a pharmaceutical composition, which is useful for the treatment and/or prevention of lipid metabolism dysfunctions associated with apolipoprotein C-III in man or animals.

More particularly, the invention relates to the use of a substance which is capable of binding to the Rev-erb receptor or to a response element thereof, for the preparation of a pharmaceutical composition which is useful for the treatment and/or prevention of lipid metabolism dysfunctions in man or animals.

The invention also relates to the use of a substance which is capable of modulating the expression

of the gene coding for the Rev-erb receptor for the preparation of a composition, in particular a pharmaceutical composition, which is useful for the treatment and/or prevention of lipid metabolism dysfunctions associated with apolipoprotein C-III in man or animals.

Among the lipid metabolism dysfunctions associated with apolipoprotein C-III in man or animals, mention may be made of hyperlipidaemia, complications associated with diabetes, obesity, syndrome X, or resistance to insulin and cardiac and coronary diseases.

A subject of the present invention is also the use of a screening process as described previously in the present patent application for the characterization, justification and claim of the mechanism of action of substances possessing anti-atherosclerotic properties, using the Rev-erb receptors and/or the response elements thereof, as well as their effect on apo C-III.

Other advantages and characteristics of the invention will emerge from the examples which follow, describing the modulation of the expression of human apo C-III by the hRev-erbα receptor.

I. METHODS

1. Cell culture

The line HepG2 (human hepatome) is from the E.C.A.C.C. (Porton Down, Salisbury, UK), while the RK13 (rabbit kidney) cells were provided by C. Lagros (laboratory of Prof. Stéhelin). These lines were maintained under standard culture conditions (Dulbecco's modified Eagle's minimum essential medium, supplemented with 10% foetal calf serum, incubation at 37°C in a humid atmosphere of 5% CO_2/95% air). The culture medium is changed every two days.

2. Construction of the recombinant plasmids

The activity of the promoter for the apo C-III gene was studied according to the standard techniques of the art using reporter genes. The constructs -1415/+24WT-CAT and 198/+24WT-CAT, which comprise fragments of the promoter for the human apo C-III gene which were cloned upstream of the CAT reporter gene, have been described previously (56). In order to exchange the CAT reporter gene of these constructs with the Luc+ reporter gene, the luciferase reporter gene Luc+ of the reporter vector pGL3 (Promega) was excised by the enzymes Sac I and BamH I and subcloned into the corresponding sites of the vector pBKCMV (Stratagene) to form the vector pBKCMV-Luc+. The CAT reporter gene of the construct -1415/+24WT-CAT was excised by the enzymes Kpn I and BamH I. Next, it was replaced with the Luc+ reporter gene obtained by digestion of the plasmid pBKCMV-Luc+ by the enzymes Bgl II and Kpn I to create the plasmid -1415/+24WT-Luc+. This was digested with the enzyme Pst I and self-religated to produce the construct -198/+24WT-Luc+. The plasmid -1415/+24WT-Luc+ was digested with Hind III to excise the apo C-III promoter. The DNA fragment obtained was then inserted into the Hind III site of the plasmids pGL3 (Promega) and pSL301 (Pharmacia) to create the constructs -1415/+24WTpGL3 and -1415/+24WTpSL301. The orientation of the insert was defined by sequencing. The construct -198/+24WTpGL3 was obtained by digesting the construct -1415/+24WTpGL3 with Pst I and religation. The construct -1415/+24WTpSL301 was partially digested with the enzyme Eco 0109I and self-religated to create the construct -108/+24WTpSL301. The fragment -108/+24 of the apo C-III promoter was excised from this construct by the enzymes Xma I and Hind III and cloned into the corresponding sites of the vector pGL3 to create the construct -108/+24WTpGL3. The fragment -82/+24 of the human apo C-III promoter was amplified by PCR using the construct -1415/+24pGL3 as matrix by means of the primers hCIIIF33 and 512. The product obtained was

digested with the enzymes Sac I and Hind III and cloned into the corresponding sites of the plasmid pGL3 to give the construct -82/+24WTpGL3. To produce the construct -64/+24WTpGL3, the construct -1415/+24pGL3 was digested exhaustively with the enzyme BstX I, made blunt by treatment with the Klenow fragment of DNA polymerase, digested with the enzyme Sma I and self-religated. To create the construct -62/+24WTpGL3, the construct -1415/+24WTpSL301 was digested exhaustively by the enzyme Eco 0109I, made blunt by treatment with the Klenow fragment of DNA polymerase and self-religated. The fragment -62/+24 of the apo C-III promoter was then excised from this construct with the enzymes Xma I and Hind III and cloned into the corresponding sites of the vector pGL3. The point mutants of the apo C-III promoter -1415/+24TaTaKOpGL3, -198/+24TaTaKOpGL3 and -82/+24TaTaKOpGL3 were obtained using the "quick change site directed mutagenesis kit" (Stratagene) according to the manufacturer's instructions, using the oligonucleotides hCIIIF29/hCIIIR29 and the corresponding wild-type constructs as matrix.

Table 1 collates the sequences of the oligonucleotides used.

Name	Sequence	5′ end	3′ end	Use	Comments
hCIIIR29	5′-CAGGCAGGAGGGTTC**ATGTG**TGTTTTATATCATCTCC-3′	-3	-39	mutagenesis	-22, -21, -20, -19, -18
hCIIIF33	5′-CCCTCATCTCCACTGGTGAGCTCGTG-3′	-106	-81	cloning	+ site Sac I
hCIIIF34	5′-GATCCGATAAAACAGGTCAGAA-3′	-33	-15	cloning, gel shift	+ site BamH I 22, -21, -20, -19, -18, mutated
hCIIIR34	5′-GATCTTCTGACCTGTTTTATCG-3′	-15	-34	cloning, gel shift	+ site Bgl II 22, -21, -20, -19, -18, mutated
hCIIIF35	5′-GATCCGATAAAACACACATGAA-3′	-33	-15	cloning, gel shift	+ site BamH I
hCIIIR35	5′-GATCTTCATGTGTGTTTTATCG-3′	-15	-34	cloning, gel shift	+ site Bgl II
hCIIIF36	5′-GATCCCGCTGGGCAAAGGTCACCTGCA-3′	-67	-90	cloning, gel shift	+ site BamH I
hCIIIR36	5′-GATCTGCAGGTGACCTTTGCCCAGCGC-3′	-90	-67	cloning, gel shift	+ site Bgl II
hCIIIF38	5′-GATCCTCACCTGCTGACCAGTGGAGA-3′	-80	-100	cloning, gel shift	+ site BamH I
hCIIIR38	5′-GATCTCTCCACTGGTCAGCAGGTGAG-3′	-100	-80	cloning, gel shift	+ site Bgl II
82	5′-GATGGGATCCGCCAGGGTTTTCCCAGTCACGAC-3′	4232	4282	cloning	pBLCAT4
510	5′-TCGCCAAGCTTCTCGTGATCTGCGGCA-3′	215	189	cloning	+ site Hind

Name	Sequence	5' end	3' end	Use	Comments
					III; pBLCAT4
512	5'-TATGCAGTTGCTCTCCAGCGGTTCCATCTTCC-3'	169	138	cloning	pGL3
514	5'-CGACTCTAGAAGATCTTGCCCCGCCCAGCG-3'	21	50	cloning	pBLCAT4
1129	5'-GATCCGGAAAAGTGTGTCACTGGGGCACGA-3'			cloning, gel shift	+ site BamH I
1142	5'-GATCTCGTGCCCCAGTGACACACTTTTCCG-3'			cloning, gel shift	+ site Bgl II

Table 1

Name	Sequence	5′ end	3′ end	Use	Comments
hCIIIF6a	5′-*GATCC*TCATCTCCACTGGTCAGCAGGTGACCTTTGC-3′	-104	-72	gel shift	
hCIIIR6a	5′-*GATC*GGCAAAGGTCACCTGCTGACCAGTGGAGATGAG-3′	-72	-104	gel shift	
hCIIIF8	5′-*GATC*TGATATAAAACAGGTCAGAACCCTC-3′	-34	-10	gel shift	
hCIIIR8	5′-*GATC*GAGGGTTCTGACCTGTTTTATATCA-3′	-10	-34	gel shift	
hCIIIF12	5′-*GATC*GATATAAAACAGG**CAG**GAACCCTC-3′	-33	-10	gel shift	-20, -19, -18 mutated
hCIIIR12	5′-*GATC*GAGGGTTC**CTG**CCTGTTTTATATC-3′	-10	-33	gel shift	-20, -19, -18 mutated
hCIIIF15	5′-*GATCC*TCAGTGCCTGCTGCCCTGGAGATGATATA*A*-3′	-56	-27	cloning, gel shift	+ site BamH I
hCIIIR15	5′-*GATCT*TATATCATCTCCAGGGCAGCAGGCACTGA*G*-3′	-27	-56	cloning, gel shift	+ site Bgl II
hCIIIF17	5′-*GATCC*TTGCCCAGCGCCCTGGGTCCTCAGTGCCTGA-3′	-76	-47	cloning, gel shift	+ site BamH I
hCIIIR17	5′-*GATCT*CAGGCACTGAGGACCCAGGGCGCTGGGCAA*G*-3′	-47	-76	cloning, gel shift	+ site Bgl II
hCIIIF21	5′-*GATCT*CATCTCCACTGGTCAGCAGGTGACCTTTGCCCAGCGCCCT*G*-3′	-102	-62	cloning, gel shift	+ site Bgl II
hCIIIR21	5′-*GATCC*AGGGCGCTGGGCAAAGGTCACCTGCTGACCAGTGGAGATGA-3′	-62	-102	cloning, gel shift	+ site BamH I
hCIIIF29	5′-GGAGATGATATAAAACA**CACAT**GAACCCTCCTGCCTG-3′	-39	-3	mutagenesis	-22, -21, -20, -19, -18

The plasmid Tk-Luc+ was constructed by inserting the Luc+ reporter gene, obtained by digesting the plasmid pBKCMV-Luc+ with the enzymes Bgl II and Kpn I, into the vector pBLCAT4 (32) cleaved with Bgl II and Kpn I, in place of the CAT reporter gene. The construct $(RevDR2)_{3x}TkLuc+$ (given as RevDR2TkLuc+ in Figure 12b) was obtained by exchanging the CAT reporter gene of the corresponding construct with the Luc+ reporter gene (Bgl II/EcoR I digestion). The corresponding CAT construct was obtained by the strategy described previously (57) using the oligonucleotides 1129 and 1142 (Table 1). The plasmid pTkpGL3 was constructed by PCR amplification of the fragment of the thymidine kinase promoter of the herpes simplex virus which is present in the plasmid pBLCAT4, using the primers 514 and 510 (Table 1). The PCR fragment obtained was then digested with the enzymes Bgl II and Hind III and inserted into the corresponding sites of the vector pGL3. The constructs $(-58/-27)_{8x}TkpGL3$ and $(-47/-79)_{1x}TkpGL3$ were obtained according to the strategy described previously (57) using the oligonucleotides hCIIIF15/hCIIIR15 and hCIIIF17/hCIIIR17, respectively. The intermediate constructs in the vector pic20H were digested with the enzymes Sal I and Xho I. The inserts obtained were then cloned into the Xho I site of the vector TkpGL3 and their orientation defined by sequencing. In order to insert, in a single step, several oriented copies of DNA fragments liable to contain the elements of response to the nuclear receptors studied according to the strategy described previously (57), the construct pTkpGL3 was digested with the enzyme BamH I, made blunt by treatment with the Klenow fragment of DNA polymerase and self-religated (vector TkpGL3BKO). The constructs $(-33/-16)_{3x}TkpGL3$, $(-33/-16TaTaKO)_{3x}TkpGL3$, $(-109/-62)_{1x}TkpGL3$, $(-100/-80)_{3x}TkpGL3$, $(-87/-67)_{3x}TkpGL3$ and $(-87/-67C3P3'KO)_{3x}TkpGL3$ were obtained by cloning into the vector TkpGL3BKO, according to the strategy described previously (57), using the oligonucleotides

hCIIIF34 and hCIIIR34, hCIIIF35 and hCIIIR35, hCIIIF21 and hCIIIR21, hCIIIF38 and hCIIIR38, hCIIIF36 and hCIIIR36, hCIIIF37 and hCIIIR37, respectively. The plasmid pG5TkpGL3 was obtained by inserting 5 copies of the response element of the yeast transcription factor Gal4 (site 17 m) (49) upstream of the Tk promoter in the plasmid TkpGL3.

The plasmids pSG5-hHNF4, pSG5-hRev-erbα, pSG5-cRev-erbβ and pCMX-hRORα1, allowing the exogenous expression of the corresponding nuclear receptors, being obtained as described previously (25, 31, 34, 35). The plasmid pGal4-ϕ was constructed by subcloning the DNA binding domain of the yeast transcription factor Gal4 present in the plasmid pBD-Gal4 (Stratagene) into the Hind III and EcoR I sites of the vector pCDNA3. In order to generate the plasmid pBDGal4-hRev-erbαDEF, the plasmid pSG5-hRev-erbα was cleaved with the enzymes Xho I and BamH I and cloned into the corresponding sites of the vector pBKCMV. The plasmid thus obtained was then digested with the enzyme Xho I, made blunt by treatment with the Klenow fragment of DNA polymerase and digested with the enzyme Spe I. This insert was then cloned into the vector pGal4-ϕ prerestricted with EcoR I, made blunt by treatment with the Klenow fragment of DNA polymerase and digested with Xba I to create the plasmid pGal4-hRev-erbαDEF. All the constructs were confirmed by sequencing.

3. Transient transfection and measurement of activity of the human apo C-III promoter

The activity of the nuclear receptors was measured by standard techniques of reporter gene/cotransfection. The DNA was introduced into the cells studied by the common techniques available in the laboratory (calcium phosphate, electroporation, lipofection, etc.). The vectors pSG5, pCDNA3 and pCMX were used as negative controls. In the experiments performed using the technique of precipitation with calcium phosphate, the cells plated out in 60 mm culture dishes

were transfected at 50-60% confluence with a plasmid mixture which generally comprised, in addition to the reporter plasmids CAT, Luc+ or pGL3 (0.5 µg/60 mm dish) and the expression vectors pSG5-hRev-erbα, pCMX-hRORα1 and pSG5-hHNF4 (0.1-1 µg/60 mm dish), 0.1 µg/60 mm dish of pCMV-β-gal plasmid (Clontech) used as a control of the transfection efficacy (36). After 5 to 6 hours, the cells were washed twice with a washing buffer (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2) and incubated for 36 hours in fresh culture medium containing 10% foetal calf serum. After transfection, the cells were lysed and the luciferase and β-galactosidase activities were measured according to standard protocols (37). For the experiments performed by lipofection, the cells were plated out in 24-well dishes at a rate of 10,000 cells per well and incubated for 16 hours at 37°C before transfection. The cells were then transfected for two hours at 37°C in a serum-free culture medium using a cationic lipid. The plasmids (reporter vectors: 50 ng/well; expression vectors: 100 ng/well, transfection-efficacy control vectors: pSV-βgal (Promega) (50 ng/well) and DNA entrainer (pBluescript (Stratagene) added to bring the amount of DNA transfected to 500 ng/well) were dissolved in serum-free DMEM supplemented with NaCl (150 mM), sodium bicarbonate (50 mM) and cationic lipid (6 nmol/µg DNA), spun down, incubated for 30 minutes at room temperature and added to the cells. After incubation for two hours, the cells were rinsed with the washing buffer described above and incubated for 36 hours in fresh culture medium containing 10% foetal calf serum. After the experiment, the cells were rinsed with washing buffer and the luciferase activity was measured using the "Dual-Luciferase™ Reporter Assay System" kit from Promega according to the manufacturer's instructions. The protein content of the extracts obtained was assayed by the Bradford technique using the "Bio-Rad Protein Assay" kit (Bio-Rad).

4. Gel retardations

The protein hRev-erbα was synthesized in vitro from the plasmid psG5-hRev-erbα by the reticulocyte lysate technique using the kit "TnT T7 quick coupled transcription/translation system" from Promega. The gel retardation experiments were carried out according to the protocol described previously (43, 44, 46) using oligonucleotides used to synthesize the double-stranded DNAs used as probes, which are described in Table 2.

Name	Sense oligonucleotide	Antisense oligonucleotide
HCIII-TaTaWT	hCIIIF8	hCIIIR8
HCIII-TaTaKO	hCIIIF12	hCIIIR12
C3P-DR2	hCIIIF6a	hCIIIR6a
Rev-DR2	1129	1142

Table 2

The double-stranded oligonucleotides were obtained by incubating 2.5 or 5 µg of the sense and antisense oligonucleotides diluted in a hybridization buffer (50 mM Tris-HCl pH 8, 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT) at 100°C for 10 min and then at 65°C for 10 min and by cooling the mixture slowly to room temperature.

The binding buffer had the following composition:

10 mM Hepes, 80 mM KCl, 5% glycerol, 10 mM DTT, 0.1 µg/µl polydIdC, 50 ng/µl herring sperm DNA, 1 µg/µl bovine serum albumin, reticulocyte lysate: 10%.

5. Animal models

The mice whose Rev-erbα gene has been destroyed by homologous recombination (Rev-erbα KO) were obtained by the team directed by Björn Vennström (Laboratory of Developmental Biology, CMB, Karolinska Institute, Stockholm, Sweden) (SV129OlaHsd background crossed with a BalbC background) (Chomez, P., Neveu, I., Mansén, A., Keisler, E., Larsson, L., Vennström, B., Arenas, E.,

submitted for publication). Björn Vennström provided us with blood samples and liver samples from (-/-) or wild-type (+/+) Rev-erbα KO transgenic mice subjected to a Chow diet. The blood and tissues were collected after fasting for 4 hours. The blood was taken from the caudal vein and the serum recovered after centrifugation at 4°C for 25 minutes at 12,000 revolutions/minute, stored at 4°C and used to analyse the lipid parameters, the lipoproteins and the apolipoproteins. After anaesthesia with CO_2, the mice were sacrificed and the tissue samples taken, frozen in liquid nitrogen and stored at -80°C for RNA analysis.

6. Analysis of the lipid parameters, lipoproteins and apolipoproteins

The serum lipids and apolipoproteins were determined by enzymatic tests adapted for microtitration plates using commercially available reagents. The levels of apo C-III in the serum were measured by immunonephelemetry using polyclonal antibodies produced in the laboratory of Prof. Fruchart. The cholesterol and triglyceride profiles of the lipoproteins were obtained by "Fast Protein Liquid Chromatography" (FPLC). The serum lipoproteins (200 µl pool of serum representative of the average) were separated by exclusion chromatography using a Superose 6HR 10/30 column (Pharmacia) at a constant flow rate (0.2 ml/minute of a phosphate buffer (10 mM, pH 7.4) supplemented with 0.01% EDTA and 0.01% NaN_3). The optical density of the effluent was measured at 280 nm. 0.27 ml fractions were collected and the total amounts of cholesterol and triglycerides present in these fractions were measured.

The extractions of hepatic RNA from transgenic mice, the preparation and hybridization of the Northern and dot blots and the measurement of the apo C-III mRNA levels were carried out according to the protocols described previously (38). The cDNAs of the clone 36B4 coding for human PO acidic ribosomal phosphoprotein (39), GAPDH (40), β-actin (41) or rat apo C-III (38)

were used as control. The cDNA probes were labelled with ^{32}P using random primers by means of the kit supplied by Boehringer Mannheim. The membranes were hybridized with 1.5×10^6 cpm/ml of each probe according to the protocol described previously (42). They were washed once with 0.5 × SSC buffer and 0.1% SDS at room temperature for 10 minutes and twice in the same buffer at 65°C for 30 minutes and then autoradiographed (X-OMAT-AR film, Kodak). The autoradiographs were analysed by densitometry (Biorad GS670 densitometer). The results were standardized relative to the levels of the mRNAs of the control probes used (42).

II. Results

1. hRev-erbα represses the activity of the human apo C-III promoter in HepG2 and RK13 cells.

When HepG2 cells are cotransfected with a plasmid which comprises the fragment (-1415/+24) of the human apo C-III promoter upstream of the luciferase reporter gene (-1415/+24WThCIIILuc+) and the plasmid pSG5-hRev-erbα which allows exogenous expression of the Rev-erbα nuclear receptor, a 50% reduction in the activity of the reporter gene is observed (Figure 1). Similar results are obtained when RK13 (rabbit kidney) cells are cotransfected with these same constructs (Figures 2, 3). This model, whose phenotype is more stable than that of the HepG2 cells, will be preferred for the characterization of the effect of hRev-erbα and of its isoforms. In addition, the effect of hRev-erbα depends on the amount of expression vector transfected (Figures 1, 3 and 4) and is independent of the transfection protocol used (precipitation of the DNA with calcium phosphate (Figures 1 to 3) or lipofection (Figures 4 and subsequent figures). Since the transfection efficacy by the second method is higher, since the amounts of DNA used can be greatly reduced and since the transfection can be carried out in the presence of an excess of inert entraining DNA, the

latter method is preferred. Lastly, the effect of hRev-erbα on the activity of the fragment -1415/+24 of the human apo C-III promoter is also observed with other reporter genes (e.g. CAT) (data not illustrated), with reporter plasmids whose skeletons differ, such as pBLCAT5 (Figures 1 and 3) or pGL3 (Figures 2, 4 and subsequent figures) or with other expression vectors such as pCDNA3 (data not illustrated): the effect of hRev-erbα is robust. The vector pGL3, which is widely used in the art, is preferred for the study hereinbelow.

These results suggest the presence of a response element to the hRev-erbα nuclear receptor in the human apo C-III promoter which is capable of reducing the activity of this promoter.

2. The effect of hRev-erbα is specific.

Figure 2 shows that the activity of the reporter gene for the vector lacking promoter (pGL3) is not affected by the exogenous expression of hRev-erbα. Furthermore, the activity of two heterologous promoters, the promoter for the thymidine kinase gene of the herpes simplex virus (noted as TkpGL3 in Figure 2), or the major late promoter of the SV40 virus (noted as pGL3 in Figure 2), is also insensitive to the action of hRev-erbα. The effect of this nuclear receptor on the promoter for the human apo C-III gene is thus specific.

3. The effect of hRev-erbα is dominant.

Several members of the superfamily of nuclear hormone receptors to which hRev-erbα belongs recognize response elements which are specific to the level of the human apo C-III promoter: HNF4, the complex PPAR/RXR, COUPTF-I and COUPTF-II bind to the site C3P (-82/-70) (47, 60, 61, 62) and modulate the activity of the human apo C-III promoter. In addition, we have observed that the nuclear receptor hRORα increases the activity of this promoter partly via the site C3P

(-82/-70) (unpublished data forming the subject of the filing of an independent PCT patent (PCT/EU99/02001)). In order to establish the extent to which hRev-erbα influences the action of other nuclear hormone receptors, RK13 cells were cotransfected with a fixed amount of reporter plasmid and plasmids allowing the exogenous expression of the hHNF4 or hRORα receptors and increasing amounts of plasmid allowing the exogenous expression of hRev-erbα. Irrespective of the nuclear receptor cotransfected, hRev-erbα reduces the activity of the reporter gene: the effect of hRev-erbα is dominant (Figures 3 and 4).

4. Identification of the molecular site of action of hRev-erbα

a. Analysis of the deletion mutants of the human apo C-III promoter

Figure 5 shows a decrease in the activity of the reporter gene when the apo C-III promoter cloned upstream of it is gradually truncated. The activity of the promoter is lost between positions -108 and -62. This region comprises the site C3P (-82/-70) whose importance in controlling the activity of the apo C-III promoter is known in the prior art (56, 60 and 62). In the experiments presented, the fragment -1415/+24 of the apo C-III promoter amplifies the activity of the Luc+ reporter gene of the plasmid pGL3 by a factor of 10. The exogenous expression of hRev-erbα reduces this activity to a level close to that of the pGL3 vector lacking promoter: the effect of hRev-erbα is powerful. It is clearly observed up to the deletion -108/+24. The results obtained with construct -62/+24 are difficult to interpret: the activity of the reporter gene is often close to that observed with the reporter pGL3, probably due to the absence of the C3P site. These results indicate the presence of at least one site of action of hRev-erbα in the portion of the human

apo C-III promoter included between positions -108 and +24.

In order to localize the hRev-erbα response element(s) present in this region of the apo C-III promoter, fragments overlapping this region (positions -33/-16, -58/-24, -76/-46, -87/-67 and -100/-80) were cloned into one or more oriented copies upstream of the TK promoter. Figure 6 shows that the activity of the construct $(-33/-16)_{3x}$TkpGL3 is reduced by hRev-erbα. The weak repression of the construct (-100/-80)3xTkpGL3 described in Figure 6 is not observed in all the experiments.

b. Analysis of the promoter by gel retardation

In order to identify the portions of the apo C-III promoter to which the hRev-erbα protein binds, overlapping double-stranded oligonucleotides were phosphorylated in the presence of ATP-γ^{32}P and incubated with the hRev-erbα protein synthesized *in vitro* (rabbit reticulocyte lysate programmed using the plasmid pSG5-hRev-erbα or with the unprogrammed lysate). The DNA/protein complexes thus obtained were then resolved on polyacrylamide gel (gel retardation method). Two specific hRev-erbα complexes were identified on the Rev-DR2 response element present on the promoter for the hRev-erbα gene used as reference. These complexes correspond to binding of the hRev-erbα receptor as a monomer or dimer to the response element (31). A specific hRev-erbα complex was identified on the fragment -34/-10 of the promoter for the human apo C-III gene and is marked with an arrow in Figure 7. This complex migrates to a molecular weight equivalent to that of the monomeric complex of hRev-erbα with the Rev-DR2 response element. The intensity of the hRev-erbα/(-34/-10) complex observed is weaker than that of the hRev-erb/(Rev-DR2) complex, which indicates lower affinity of the site (-34/-10) for hRev-erbα. Analysis of the sequence of the fragment -34/-10 shows the presence of a perfect AGGTCA half-site preceded by

an A/T-rich region in position -23/-18. However, the base located in position -1 relative to this half-site is a C, which differs from the consensus defined by the art. This difference may explain the low affinity of the site for hRev-erbα. The corresponding double-stranded oligonucleotide whose -23/-18 site sequence is mutated (AGGTCA→AGGCAG) (hCIIITaTamut) does not form a complex with the hRev-erbα protein (data not illustrated). Finally, we observed no significant gel retardations with labelled oligonucleotides which cover other fragments of the portion between the positions -198 and +24 of the promoter for the human apo C-III gene (for example with the double-stranded oligonucleotide corresponding to the fragment -104/-72 ("C3P-DR2") of the apo C-III promoter (Figure 7).

In conclusion, the gel retardation experiments identified the AGGTCA half-site present in position -23/-18 of the promoter for human apo C-III as a probable hRev-erbα response element.

c. Analysis of the point mutants of the promoter for the human apo C-III gene

In order to validate the results obtained with the deletion mutants and with the gel retardation technique, the constructs -1415/+24WTpGL3 and -82/+24WTpGL3 were mutated by site-directed mutagenesis on the AGGTCA half-site present downstream of the TaTa box of the apo C-III gene (-23/-18). Moreover, we cloned, upstream of the Tk promoter, three copies of the -33/-16 fragment of the human apo C-III promoter whose AGGTCA site was modified in accordance with the mutations of the constructs -1415/+24WTpGL3 and -82/+24WTpGL3. Figure 8A indicates that the mutation of the AGGTCA half-site present in position (-23/-18) of the human apo C-III promoter reduces the sensitivity of the entire promoter to hRev-erbα by 50%. The effect of hRev-erbα is totally lost when the construct -82/+24WTpGL3 is mutated. Similarly, mutation of the -23/-18 site in the construct $(-33/-16WT)_{3x}$TkpGL3 (to

give the construct $(-33/-16KO)_{3x}$TkpGL3) suppresses its sensitivity to hRev-erbα (Figure 8B).

d. Conclusions

At least one site involved in the action of hRev-erbα on the promoter for the human apo C-III gene has been clearly identified: the AGGTCA half-site located in position -23/-18 of the apo C-III promoter.

5. Effects of the hRev-erbα isoforms

Figure 9 shows, surprisingly, that the β and γ Rev-erb isoforms also repress the activity of the construct -198/+24WTLuc+.

6. Disruption of the Rev-erbα gene in Rev-erb KO mice affects the hepatic expression of apo C-III and the plasma levels of apo C-III and triglycerides

In order to establish the physiological relevance of the observations made in vitro described above, the effect of the destruction by homologous recombination of the Rev-erbα gene in SV129XBalbC mice was evaluated on the blood parameters (plasma level of triglycerides and of apo C-III, lipid profile) and the accumulation of messenger RNAs coding for apo C-III in the liver of normal and transgenic animals.

a. Blood parameters

A significant increase (Mann-Whitney test, $p < 0.05$) in the triglyceride concentration in the serum was observed in mutant mice compared with normal mice (Figure 10A). The FPLC profile indicates a large increase in triglycerides in the VLDL fraction (Figure 11).

b. Expression of the apo C-III gene

Expression of the mRNA coding for apo C-III is increased in mice whose Rev-erbα gene has been destroyed by homologous recombination (Figure 10C).

This increased expression is associated with a significant increase (Mann-Whitney test, $p < 0.05$) in the level of apo C-III in the plasma (Figure 10B).

These results show that modifications in the expression of Rev-erb affect the hepatic expression of apo C-III and the levels of triglycerides and apo C-III in the plasma in mice: our observations made *in vitro* are physiologically relevant.

7. Relevance of the screening processes proposed

Repression (Figures 1 to 5, 8 and 9) of the expression of the reporter gene cloned downstream of the promoter for the human apo C-III gene when the exogenous expression of hRev-erbα is artificially increased is the basis for the relevance of using this method to identify substances liable to modulate the activity of hRev-erbα.

Figures 6 and 12 are the basis for the relevance of using isolated sites cloned upstream of the Tk promoter before a reporter gene in order to identify substances liable to modulate the activity of hRev-erbα. A construct comprising three copies of the Rev-DR2 site present in the promoter for the human Rev-erbα gene which are cloned before the Tk promoter has been characterized (Figure 12). Its sensitivity to hRev-erbα is increased. This justifies its value for the screening of substances liable to modulate the activity of the native hRev-erbα nuclear receptor.

Lastly, Figure 13 is the basis for the relevance of using chimeras which combine the DNA binding domain of the yeast transcription factor Gal4 and the binding domain of the hRev-erbα ligand and of a reporter vector which comprises 5 copies of the Gal4 response element in order to identify substances liable to modulate the activity of hRev-erbα.

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CLAIMS

1. Use of Rev-erb receptors and/or the response element thereof or a functional equivalent of these receptors to screen substances which are useful in the treatment of lipid metabolism dysfunctions.

2. Use according to Claim 1, characterized in that the Rev-erb receptor and the Rev-erb receptor response element are the hRev-erbα receptor and the hRev-erbα receptor response element.

3. Process for screening substances which are useful in the treatment of lipid metabolism dysfunctions, characterized:

- in that the test substance is placed in contact with a receptor of the Rev-erb family and/or a Rev-erb receptor response element, and/or a nuclear factor capable of functionally coupling Rev-erb to the RNA-polymerase complex, or a functional equivalent thereof,

- in that the following are measured by any appropriate means:

- the binding of the said substance to the Rev-erb receptor or the binding of the complex formed from the said substance and the Rev-erb receptor to its response element and/or to a nuclear factor capable of functionally coupling Rev-erb to the RNA-polymerase complex, and/or

- the modulation of the transcriptional activity of the genes placed under the control of a promoter comprising the Rev-erb response element.

4. Process for screening substances which are useful in the treatment of lipid metabolism dysfunctions, which consists in determining the effect of the test substance on the modulation of the expression of the gene coding for the Rev-erb receptor.

5. Use of a substance selected by a screening process according to either of Claims 3 and 4, for the preparation of a composition, in particular a pharmaceutical composition, which is useful for the

treatment of lipid metabolism dysfunctions associated with apolipoprotein C-III in man or animals.

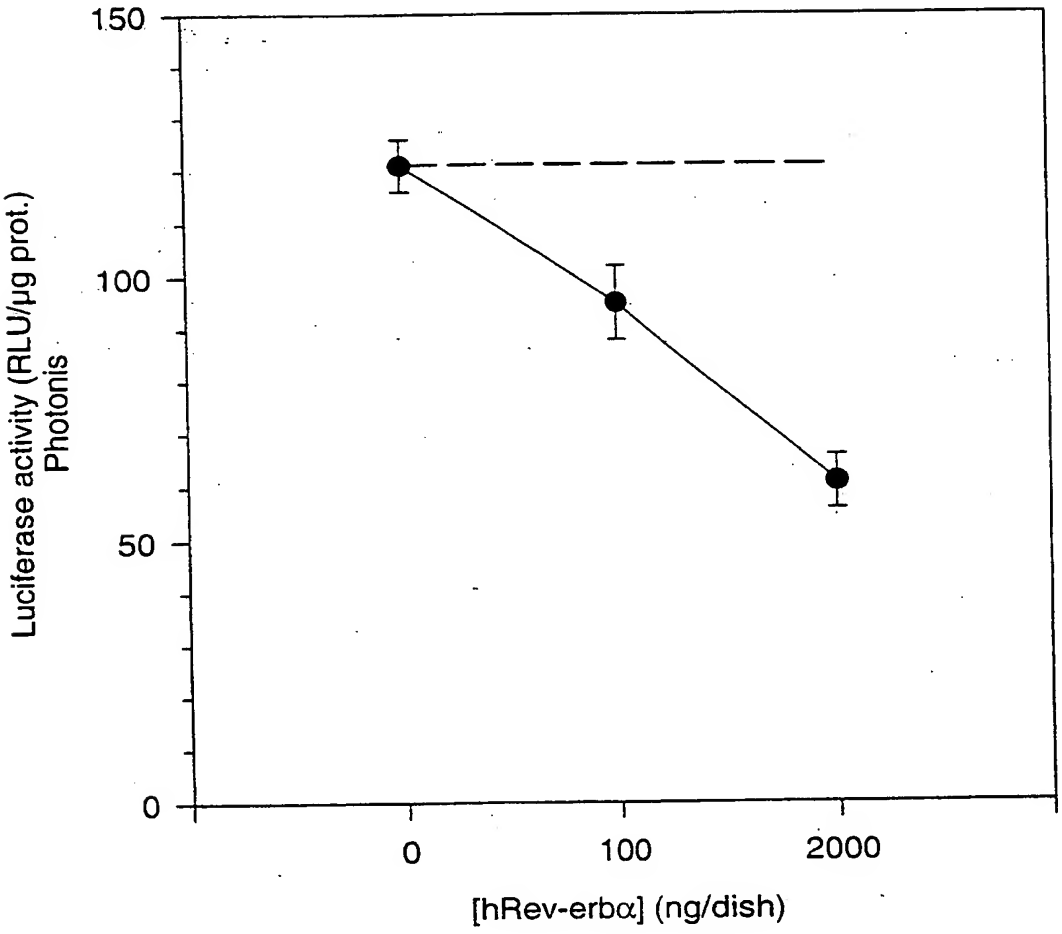
6. Use of a substance which is capable of binding to the Rev-erb receptor or to the response element thereof, for the preparation of a pharmaceutical composition which is useful for the treatment and/or prevention of lipid metabolism dysfunctions associated with apolipoprotein C-III in man or animals.

7. Use of a substance which is capable of modulating the transcriptional activity of a gene placed under the control of a promoter comprising the Rev-erb receptor response element, for the preparation of a pharmaceutical composition which is useful for the treatment and/or prevention of lipid metabolism dysfunctions associated with apolipoprotein C-III in man or animals.

8. Use of a substance which is capable of modulating the expression of the gene coding for the Rev-erb receptor for the preparation of a composition, in particular a pharmaceutical composition, which is useful for the treatment and/or prevention of lipid metabolism dysfunctions associated with apolipoprotein C-III in man or animals.

9. Use of a screening process according to either of Claims 3 and 4, for the characterization, justification and claim of the mechanism of action of substances possessing anti-atherosclerotic properties using the Rev-erb receptors and/or the response elements thereof, as well as their effect on apo C-III.

Figure 1



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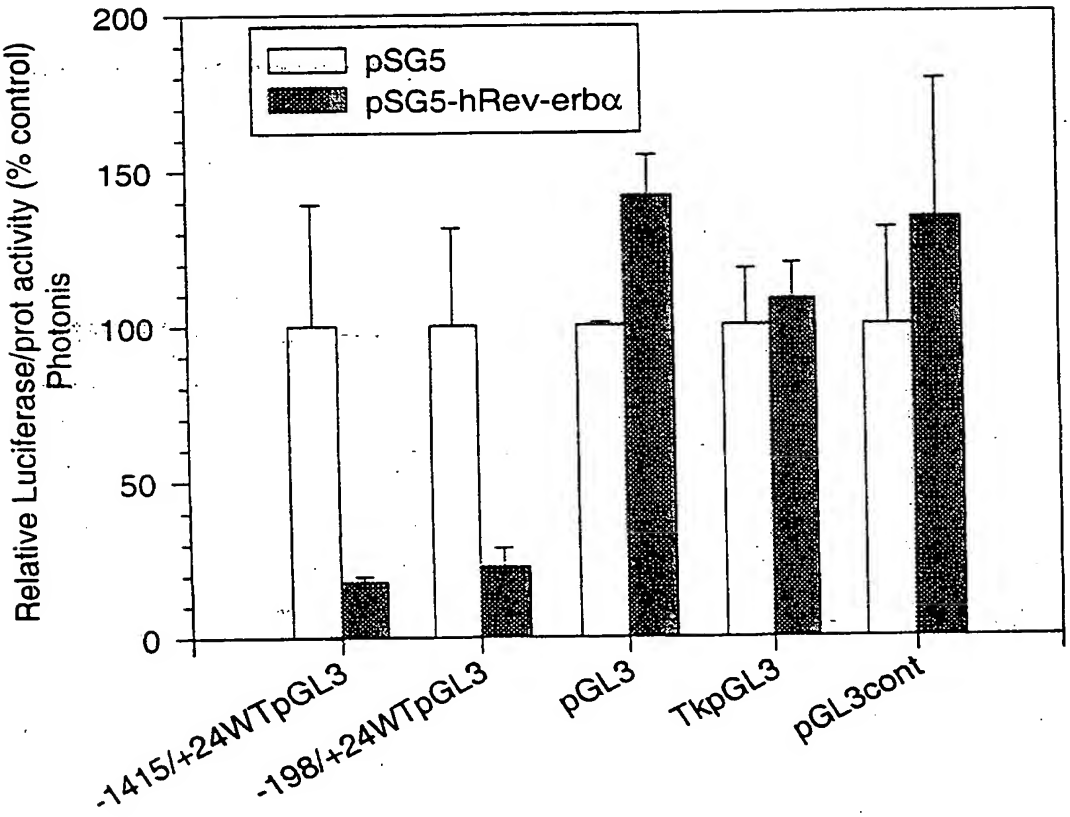

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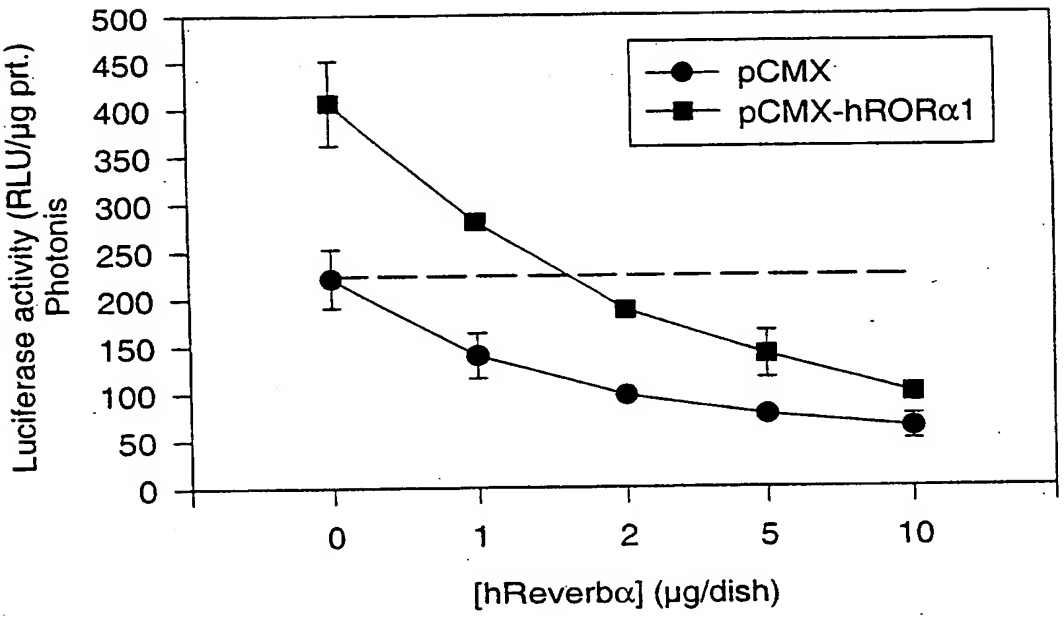

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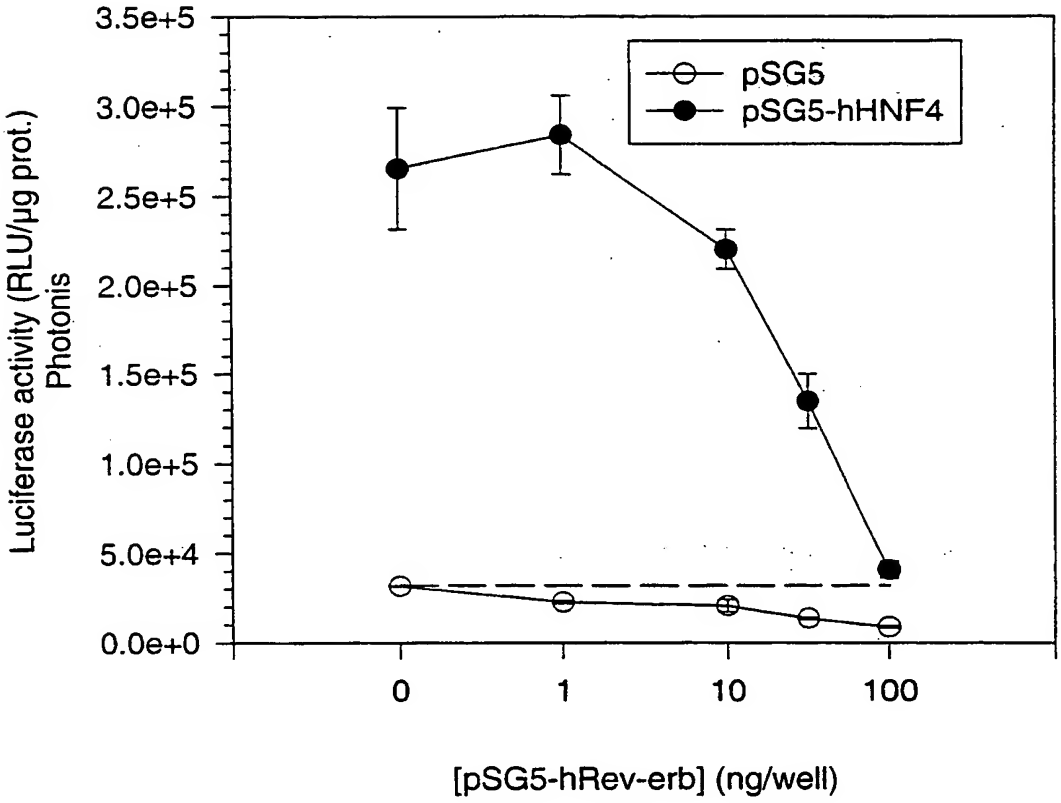

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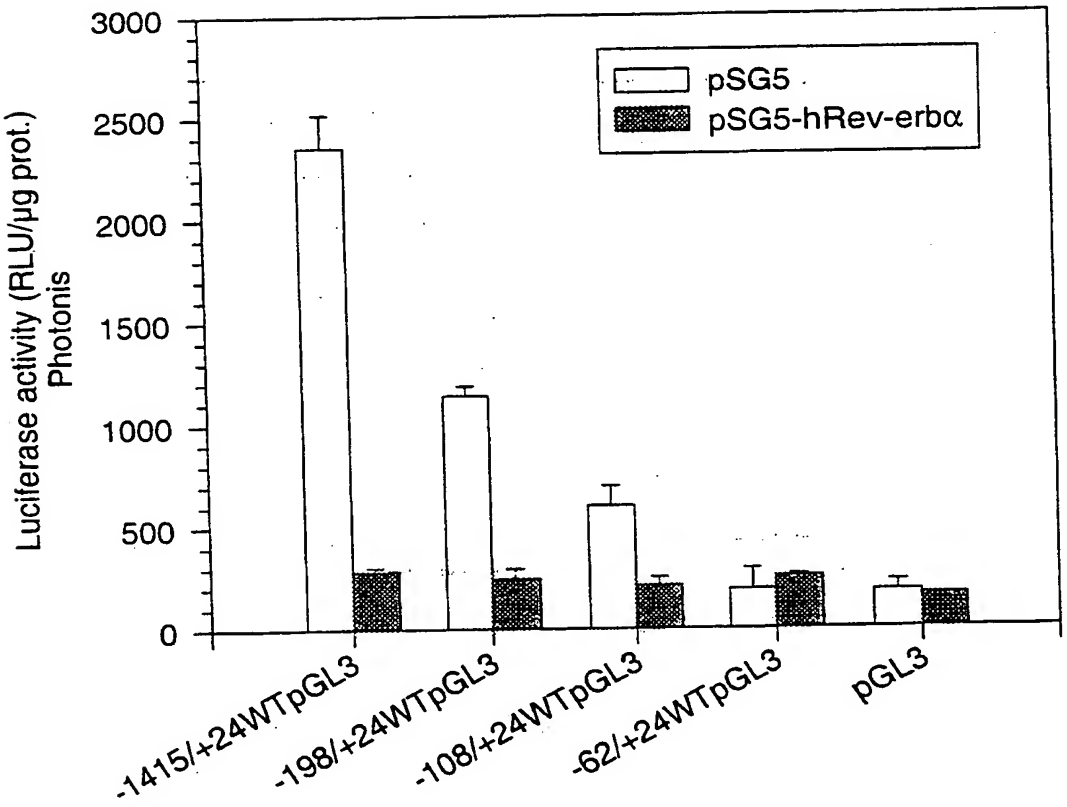

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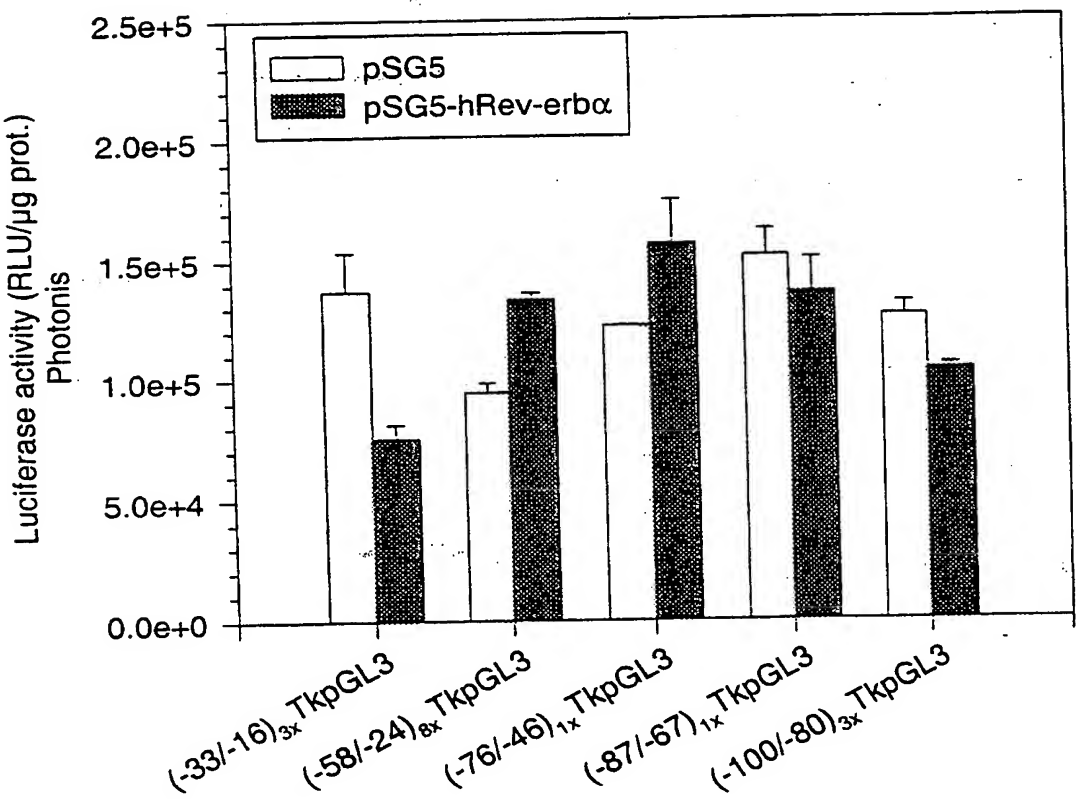

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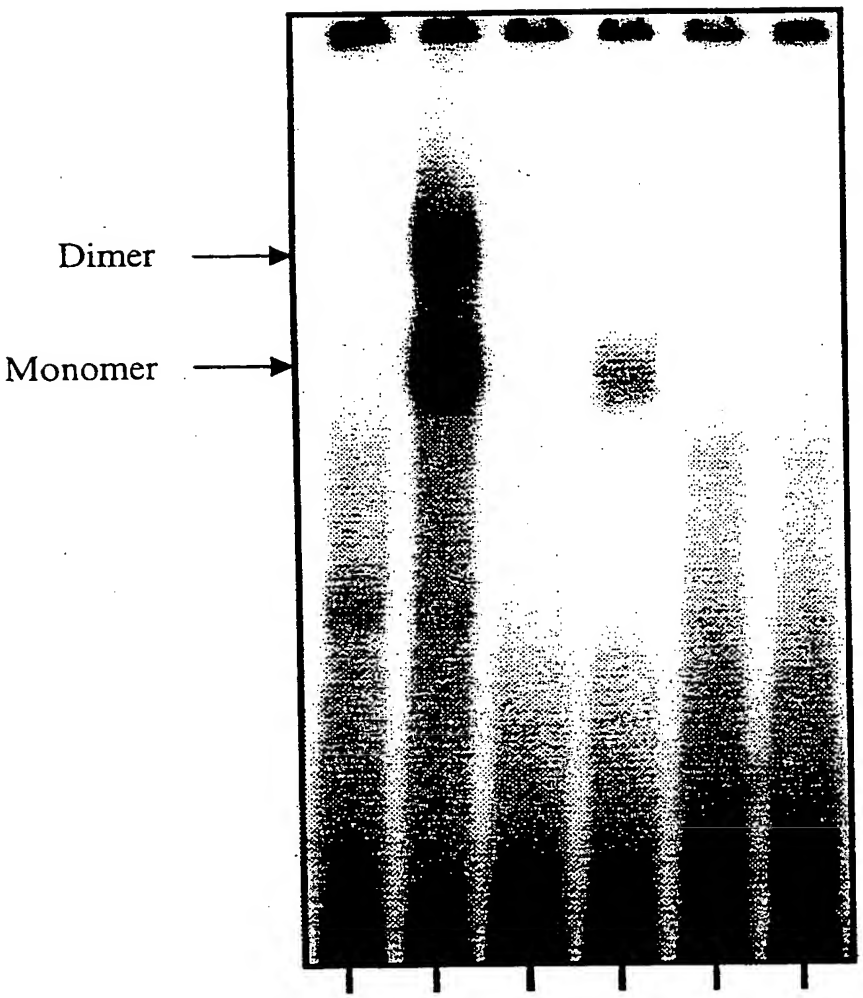

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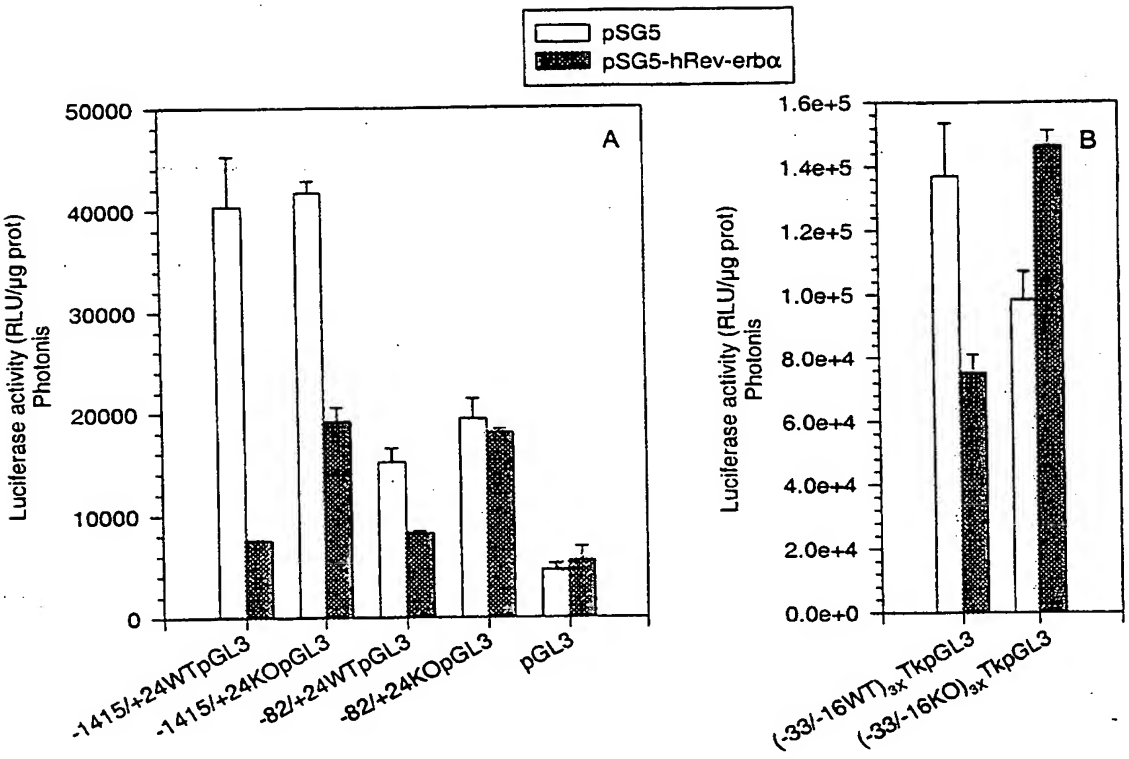

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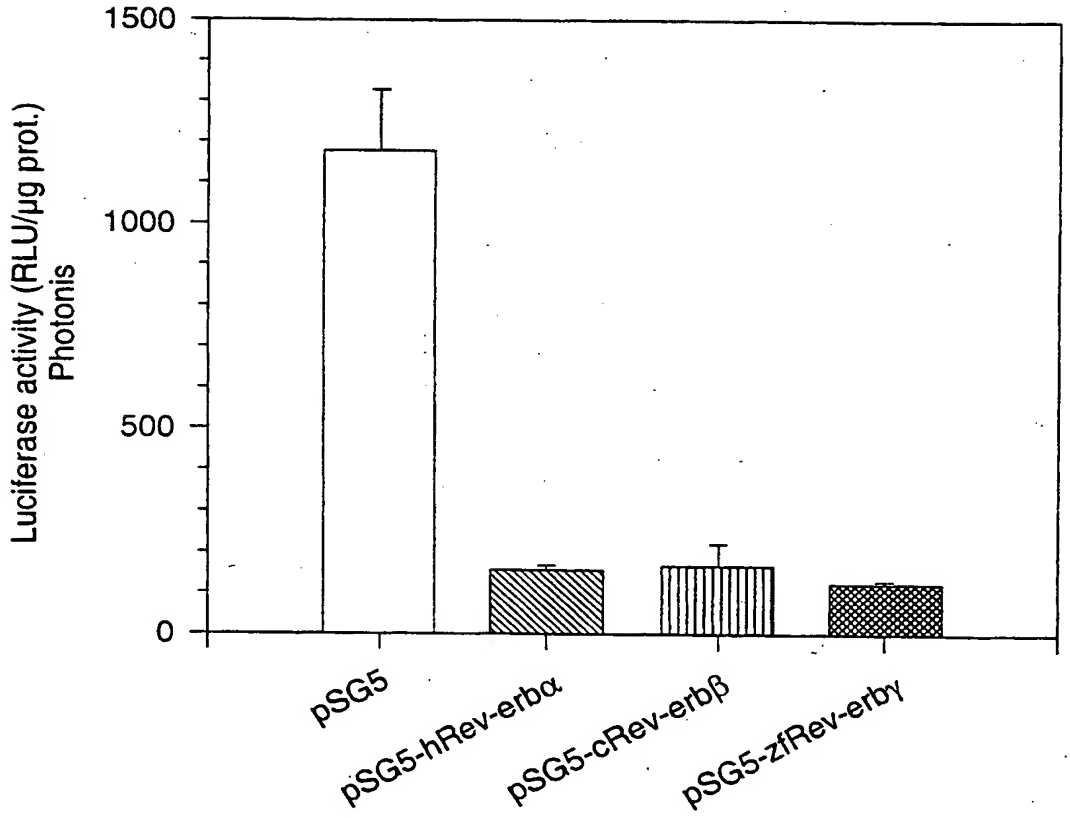
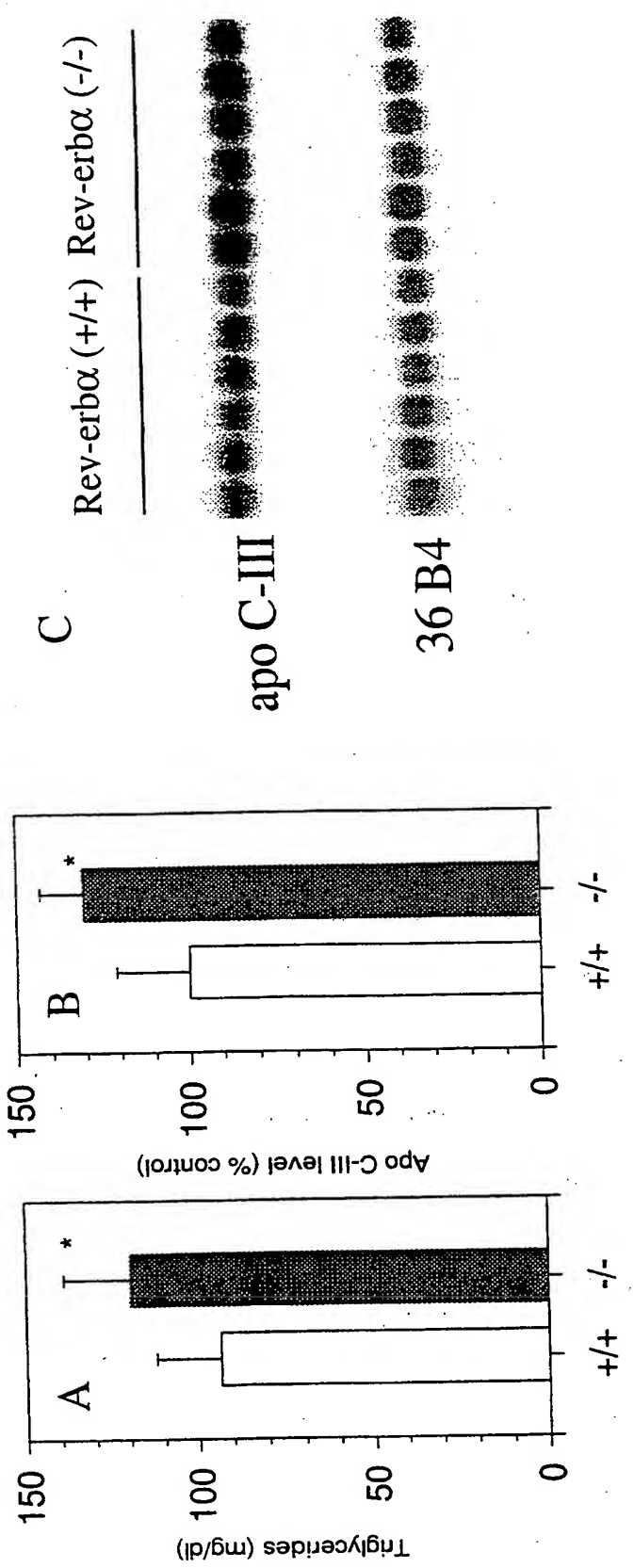


Figure 10



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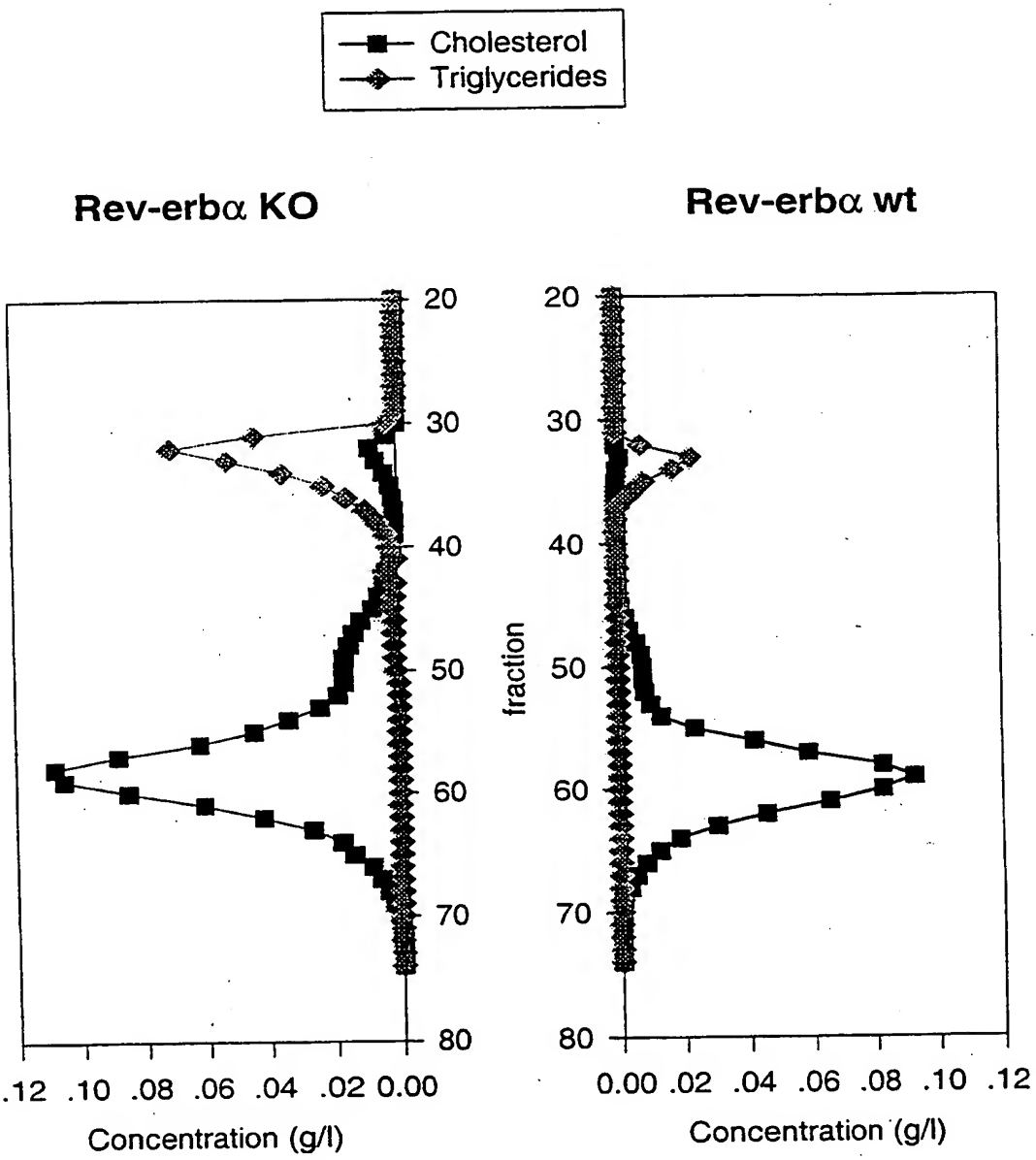
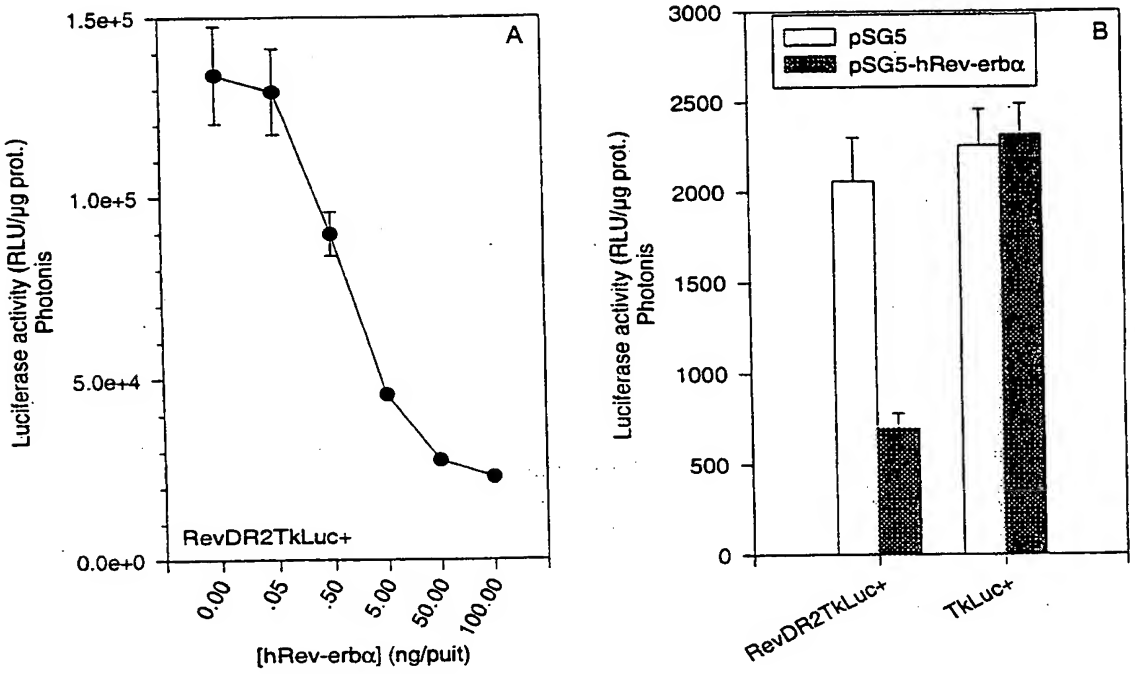


Figure 12



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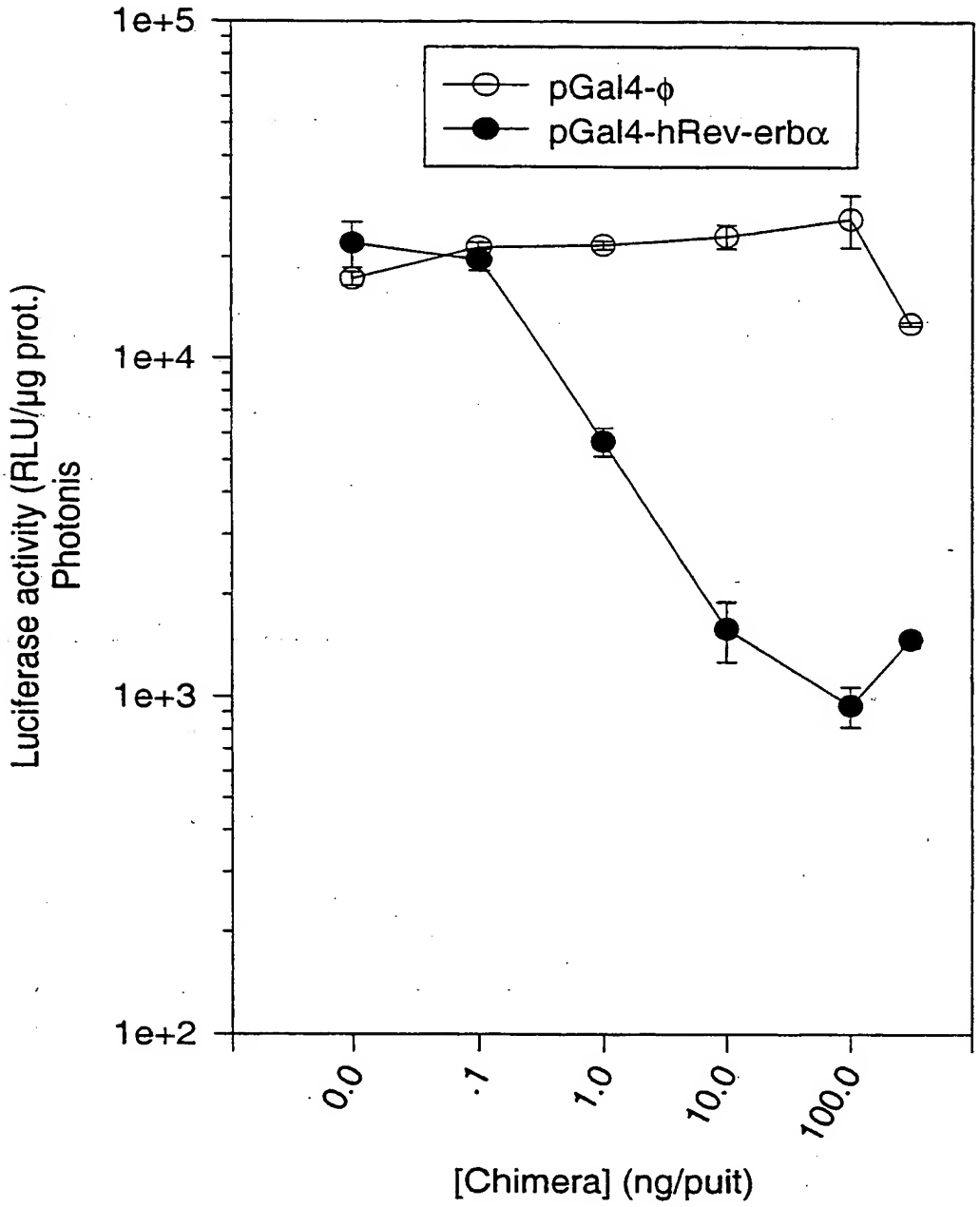

INTERNATIONAL SEARCH REPORT

International Application No
PC./EP 99/04286

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	WO 98 52968 A (SALK INST FOR BIOLOGICAL STUDI) 26 November 1998 (1998-11-26) the whole document	1-8
Y	WO 97 12853 A (LIGAND PHARM INC) 10 April 1997 (1997-04-10) claims page 2, line 14 - line 28	1-8
Y	WO 97 08550 A (SALK INST FOR BIOLOGICAL STUDI) 6 March 1997 (1997-03-06) claims page 3, line 18 - line 26 page 8, line 10 -page 9, line 26	1-8
Y	WO 96 41013 A (LIGAND PHARM INC) 19 December 1996 (1996-12-19) claims	1-8

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
3 November 1999	12/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Routledge, B

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/04286

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VU-DAC, NGOC ET AL: "The nuclear receptors peroxisome proliferator-activated receptor alpha an Rev - erbalpha mediate the species-specific regulation of apolipoprotein A-I expression by fibrates." JOURNAL OF BIOLOGICAL CHEMISTRY, (OCT. 2, 1998) VOL. 273, NO. 40, PP. 25713-25720. ISSN: 0021-9258., XP002099903 cited in the application	1-4
Y	the whole document	5-8

Y	ADELMANT ET AL: "A functional Rev-erbalpha responsive element located in the human Rev-erbalpha promoter mediates a repressing activity" PROC.NATL.ACAD.SCI. USA, vol. 93, April 1996 (1996-04), pages 3553-3558, XP002099904 cited in the application the whole document	1-8

Y	LAVRENTIADOU ET AL: "Modulation of the ApoCIII promoter activity by heterodimers of ligand dependent nuclear receptors RXR -alpha- RAR -alpha, RXR -alpha-T3R-beta and RXR -alpha-PPAR-alpha" CIRCULATION, vol. 92, no. 8, 13 November 1995 (1995-11-13), page 129 XP002088668 the whole document	1-8

Y	JANUZZI ET AL: "Characterization of the mouse apolipoprotein Apoa-1/Apoc-3 gene locus: Genomic, mRNA, and protein sequences with comparisons to other species" GENOMICS, vol. 14, no. 4, December 1992 (1992-12), pages 1081-1088, XP002088670 the whole document	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 99/04286

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9852968 A	26-11-1998	NONE	
WO 9712853 A	10-04-1997	AU 7074296 A	09-04-1997
		AU 7362496 A	28-04-1997
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		EP 0832295 A	01-04-1998

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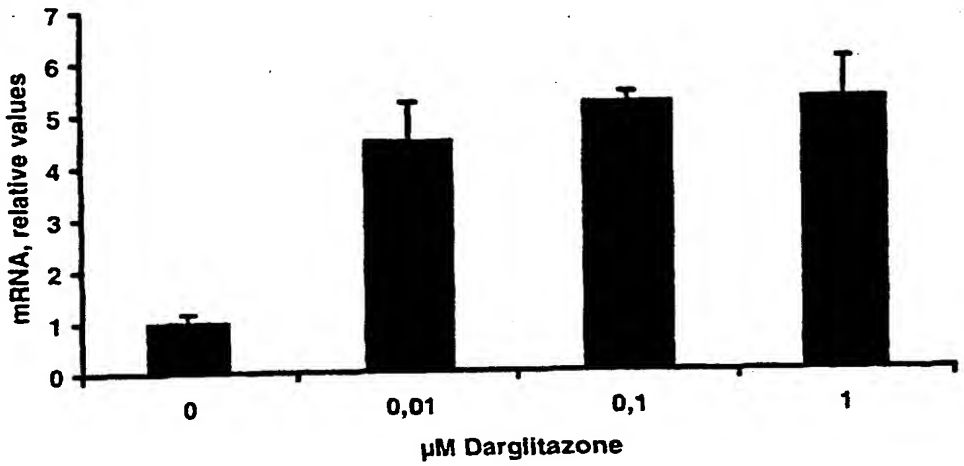

(57) Abstract: The invention also relates to the use of active modulators of LXRα activity or expression in stimulation of pre-adipocyte differentiation and hence also in the treatment of insulin resistance syndrome, or dyslipidemia, or type 2 diabetes.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

THERAPY

FIELD OF THE INVENTION

The invention relates to methods of screening test compounds for their ability to stimulate pre-adipocyte differentiation by measuring their activity as a modulator of LXRα activity or expression. The invention also relates to the use of active modulators of LXRα activity or expression in stimulation of pre-adipocyte differentiation and hence also in the treatment of insulin resistance syndrome, or dyslipidemia, or type 2 diabetes.

BACKGROUND OF THE INVENTION

PPARγ is an established master switch for driving adipocyte differentiation. Retrovirus-mediated expression of PPARγ in a fibroblast cell line (NIH-3T3) conferred an adipocyte phenotype onto this otherwise non-adipogenic cell (Tontonoz et al., 1994). Treatment of 3T3-L1 pre-adipocytes with Pioglitazone (a PPARγ agonist of the thiazolidinedione class) enhanced the insulin or insulin-like growth factor-1 (IGF-I)-regulated differentiation as monitored by the rate of lipogenesis or triglyceride accumulation (Kletzien et al., 1992). Pioglitazone caused both a leftward shift and enhanced maximum response for the IGF-I-regulated differentiation of the cells, consistent with the idea that the drug enhances the sensitivity of cells to polypeptide hormones. PPARγ agonists are therefore promoters of adipocyte differentiation and insulin sensitisers and are prescribed clinically to treat type 2 diabetes.

Here we show that a thiazolidinedione, Darglitazone, leads to increased expression of the nuclear receptor LXRα in 3T3-L1 adipocytes and in human primary adipocytes. In addition we show that activation of LXRα leads to differentiation of pre-adipocyte cells to adipocytes.

The LXRs were first identified as orphan members of the nuclear receptor superfamily (Willy et al., 1995) and have later been shown to be activated by a specific class of naturally occurring, oxidised derivatives of cholesterol, including 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol (Janowski et al., 1996, Janowski et al., 1999). Two members of the LXR family have been identified: the tissue restricted (mainly liver, intestine, kidney and adipocytes) LXRα and the ubiquitous LXRβ (Peet et al., 1998, Repa & Mangelsdorf, 1999). When cholesterol is in excess and its oxidised metabolites are

present, LXRα is activated and induces transcription of the Cyp7a1 gene, which encodes the rate-limiting enzyme in the classical bile acid synthesis pathway cholesterol 7α-hydroxylase. Upregulation of cholesterol 7α-hydroxylase enhances conversion of cholesterol to bile acids, thereby reducing the amount of circulating cholesterol. The role of LXRα as a key regulator of cholesterol homeostasis has been studied in mice homozygous for a disrupted LXRα gene. These genetically modified mice are apparently healthy and fertile when fed with a normal diet. However, when given a high content cholesterol diet (0,2% or 2%), hepatomegaly with cholesterol accumulation occurs, leading to hepatic failure, and also failure of Cyp7a1 transcription induction was detected. These results provide evidence that LXRα is required to regulate Cyp7a1 expression in mice and that this is very important for maintenance of cholesterol homeostasis. These observations have led to the suggested use of LXRα agonists to increase the synthesis of bile acids as a means to lower the level of blood cholesterol.

Recently the gene encoding the ATP-binding cassette transporter protein 1 (ABC-1), was reported to be transcriptionally regulated by LXRα (Costet et al., 2000, Repa et al., 2000). The ABC-1 transporter is involved in cellular efflux of cholesterol to high density lipoproteins (HDL). Interestingly, several genetic defects in this transporter are also characterised by accumulation of cholesterol in various tissues and increased risk of coronary artery disease in patients belonging to a Tangiers disease cohort. This indicates that LXRα may have additional roles in the regulation of cholesterol levels besides controlling the Cyp7a1 gene.

WO 93/06215 (EP609240), The Salk Institute. This application describes the cloning of five new orphan receptors belonging to the steroid/thyroid superfamily of receptors, one (designated XR2) has later been shown to be the human LXRα.

WO 96/21726, The Salk Institute. This application describes the characterisation of LXRα and claims certain response elements, LXR/RXR heterodimers, and LXR based assays.

WO 99/18124 (EP1021462) Merck & Co. This application covers methods for identifying agonist and antagonists of nuclear receptors. The claimed methods comprises the use of a nuclear receptor or a ligand binding domain thereof labelled with a first fluorescent reagent; a nuclear receptor co-activator or a binding portion thereof labelled with a second fluorescent reagent; and measuring FRET between the first and second fluorescent reagents. LXR is exemplified as one of the nuclear receptors of the claimed methods and SRC-1 as a co-activator.

WO 00/34461 University of Texas. This application covers various aspects of modulating cholesterol metabolism, such as LXRα knock-out mice and their use in screens, screen for LXRα agonists for their ability to increase bile acid synthesis, screening for substances reducing cholesterol levels or increasing bile acid synthesis using LXRα knock-outs, screen for modulators of ABC1 expression.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that agonists of LXRα activity stimulate differentiation of pre-adipocytes. In addition, differentiation of a pre-adipocytes is accompanied by an increased expression of LXRα . Stimulation of differentiation of pre-adipocytes is useful also in the treatment of insulin resistance syndrome, or dyslipidemia, or type 2 diabetes.

In one aspect, the invention features a method of stimulating pre-adipocyte differentiation in a cell comprising administering a LXRα agonist to a cell, wherein the agonist stimulates pre-adipocyte differentiation. In one embodiment, the cell is a mammalian cell such as an adipocyte cell, a 3T3-L1 pre-adipocyte cell, or a 3T3-L1 adipocyte cell. In one embodiment, the LXRα agonist is an oxidized derivative of cholesterol such as 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol. In another embodiment, the LXRα agonist is a thiazolidinedione compound such as darglitazone, rosiglitazone, pioglitazone, or troglitazone, and their pharmaceutically acceptable salts.

In another aspect, the invention features a method of treating a disorder associated with aberrant pre-adipocyte differentiation. The method includes administering a therapeutically effective amount of a LXRα modulator to a mammal, wherein the LXRα modulator stimulates pre-adipocyte differentiation. In one embodiment the LXRα modulator is an oxidized derivative of cholesterol such as 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol. In another embodiment, the LXRα modulator is a thiazolidinedione compound such as darglitazone, rosiglitazone, pioglitazone, or troglitazone, and their pharmaceutically acceptable salts. The disorder can be any disorder which has an aberrant adipocyte differenentiation, e.g., the disorder can be insulin resistance syndrome, dyslipidemia or type 2 diabetes. The LXRα modulator can be administered in any manner known in the art including orally, topically, intravenously, transdermally, rectally, or parentally. In one embodiment the modulator is administered to the mammal in a

pharmaceutical composition comprising a pharmaceutically acceptable carrier or excipient.

In another aspect the invention features a method of increasing the level of LXRα expression or activity, comprising administering a pharmaceutically effective amount of a LXRα modulator. In one embodiment the LXRα modulator is an oxidized derivative of cholesterol such as 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol. In another embodiment, the LXRα modulator is a thiazolidinedione compound such as darglitazone, rosiglitazone, pioglitazone, or troglitazone, and their pharmaceutically acceptable salts In one embodiment the modulator is administered to a pre-adipocyte cell in a mammal. In another embodiment the mammal has insulin resistance syndrome, dyslipidemia or type 2 diabetes.

The invention further relates to the use of a variety of procedures for using the LXRα receptor in the discovery of modulators of the receptor function or expression, such modulators may be used in stimulating pre-adipocyte differentiation and therefore used to modify or ameliorate insulin resistance syndrome or dyslipidemia or type 2 diabetes.

In one aspect, the invention features a method for identifying a compound that stimulates pre-adipocyte differenciation. The method includes providing a cell comprising a LXRα regulatory sequence operatively linked to a reporter gene; introducing a test compound into the cell; assaying for transcription of the reporter gene in the cell, wherein an increase in transcription in the presence of the compound compared to transcription in the absence of the compound indicates that the compound stimulates pre-adipocyte differenciation. The cell can be any cell such as a mammalian cell. In one embodiment the cell is an adipocyte cell, a 3T3-L1 pre-adipocyte cell, or a 3T3-L1 adipocyte cell. The reporter gene can encode a luciferase, a chloramphenicol acetyl transferase, a beta-galactosidase, an alkaline phosphate, or a fluorescent protein.

In another aspect, the invention features a method of identifying a compound which binds to a LXRα polypeptide comprising contacting a LXRα polypeptide, or a cell expressing a LXRα polypeptide, with a test compound; and determining if the polypeptide binds to the test compound. The binding of the test compound to the polypeptide can be detected by direct detecting of the compound to the polypeptide or by a competition binding assay.

The invention further features a method for identifying a compound which modulates the activity of a LXRα polypeptide comprising contacting a LXRα polypeptide with a test compound and assaying for the ability of the test compound to stimulate pre-adipocyte

differentiation, wherein an increase in the ability of the polypeptide to stimulate pre-adipocyte differentiation indicates that the compound modulates the activity of the LXRα polypeptide.

In another aspect, the invention features a method of identifying an agonist of LXRα which includes contacting a LXRα protein, or fragment thereof, a LXRα coactivator and a compound; and determining if the LXRα protein, or fragment thereof, and the LXRα coactivator interact, wherein an interaction between the LXRα protein, or fragment thereof, and the LXRα coactivator indicates that the compound is a LXRα agonist. In one embodiment the LXRα co-activator is a steroid receptor co-activator.

In yet another aspect, the invention features a method of identifying an agonist of LXRα which includes contacting a LXRα protein, or fragment thereof, a LXRα heterodimerization partner or fragment thereof, and a compound; and determining if the LXRα protein, or fragment thereof, and the LXRα heterodimerization partner, or fragment thereof, interact, wherein an interaction between the LXRα protein, or fragment thereof, and the LXRα heterodimerization partner, or fragment thereof, indicates that the compound is a LXRα agonist. In one embodiment, the LXRα heterodimerization partner is a retinoid X receptor.

The invention relates to pharmaceutical compositions containing such a modulator discovered by the methods described in this application and the use of the modulator or pharmaceutical composition comprising such modulator in stimulating pre-adipocyte differentiation and therefore used to modify or ameliorate insulin resistance syndrome or dyslipidemia or type 2 diabetes.

In one aspect the invention feature the use of a LXRα modulator in the manufacture of a medicament for the treatment of a disorder associated with aberrant pre-adipocyte differentiation, wherein the LXRα modulator stimulates pre-adipocyte differentiation. In one embodiment the LXRα modulator is an oxidized derivative of cholesterol such as 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol. In another embodiment the LXRα modulator is a thiazolidinedione compound such as darglitazone, rosiglitazone, pioglitazone, or troglitazone, and their pharmaceutically acceptable salts. The disoder can be any disorder associated with aberrant pre-adipocyte differentiation such as insulin resistance syndrome, dyslipidemia or type 2 diabetes. The LXRα modulator can be administered orally, topically, intravenously, transdermally, rectally, or parentally.

In yet another aspect the invention features a pharmaceutical formulation for use in the

treatment of a disorder associated with aberrant pre-adipocyte differentiation. In one embodiment the LXRα modulator is an oxidized derivative of cholesterol such as 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol. In another embodiment the LXRα modulator is a thiazolidinedione compound such as darglitazone, rosiglitazone, pioglitazone, or troglitazone, and their pharmaceutically acceptable salts. The disoder can be any disorder associated with aberrant pre-adipocyte differentiation such as insulin resistance syndrome, dyslipidemia or type 2 diabetes. The LXRα modulator can be administered orally, topically, intravenously, transdermally, rectally, or parentally.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts a bar chart showing Northern blot anaylsis of total RNA isolated from differentiated 3T3-L1 cells probed with an LXRα probe.

Figure 2 depicts a bar chart showing Northern blot analysis of 20 µg total RNA obtained from fully differentiated 3T3-L1 cells stimulated with 22-R (5 µM), Darglitazone (1 µM) alone or in combination for 24 hours, probed with an LXRα probe.

Figure 3 depicts a bar chart showing Northern blot analysis of polyA+ RNA isolated from human adipocytes grown in the presence of 1 µM Darglitazone (Dar), 5 µM 22-R-hydroxy cholesterol (22-R-OH) or both (Dar + 22-R-OH), probed with an LXRα probe.

DETAILED DESCRIPTION OF THE INVENTION

Treatment of 3T3-L1 pre-adipocytes with an LXRα agonist, 22-R-hydroxycholesterol, enhances adipocyte differentiation of 3T3-L1 pre-adipocytes. This demonstrates that LXRα not only is very important for maintenance of cholesterol homeostasis but represents an important regulatory factor in adipocyte differentiation. Treatment with an LXRα modulator of activity or expression can lead to stimulation of pre-adipocyte differentiation and have utility in improving insulin sensitisation and therefore constitutes a novel treatment for dyslipidemia and insulin resistance and type 2 diabetes.

This invention provides a method for stimulation of pre-adipocyte differentiation comprising the administration of an effective amount of a modulator of the activity or expression of LXRα to a patient in need of such treatment.

Modulation, preferably by an "upregulator") of the expression of LXRα by a compound may be brought about, for example, through altered gene expression levels or message stability. Modulation, preferably by an "agonist", of the activity of LXRα by a compound may be brought about for example through compound binding to LXRα, LXRα /RXRα heterodimer, LXRα/co-activator or LXRα/RXRα/co-activator complexes.

In a further aspect of the present invention we provide a method for the provision of an adipocyte differentiation agent, which method comprises using one or more putative modulator of LXRα expression or activity as test compounds in one or more procedure to measure the ability of the test compound to modulate LXRα, and selecting an active compound for use as an agent able to stimulate pre-adipocyte differentiation.

Convenient test procedures include the use of animal models to test the role of the test compound. These will typically involve the administration of compounds by intra peritoneal injection, subcutaneous injection, intravenous injection, oral gavage or direct injection via canullae into the blood stream of experimental animals. The effects on insulin sensitivity, lipid profiles, food intake, body temperature, metabolic rate, behavioural activities and body weight changes may all be measured using standard procedures.

Suitable modulators may be firstly identified by screening against the isolated LXRα receptor or fragment or chimeirc form thereof.

Preferably the screen is selected from:

i) measurement of LXRα activity using a reporter gene assay comprising a cell line which expresses LXRα and a reporter gene coupled to an LXRα response element and assaying for expression of the reporter gene.

ii) measurement of LXRα activity using purified LXRα protein or a fragment thereof and a co-activator or a fragment thereof, and assaying the interaction between LXRα and the co-activator, preferably by time resolved fluorescence resonance energy transfer or by scintillation proximity assay.

iii) measurement of LXRα activity using purified LXRα protein or a fragment thereof and a heterodimerization partner or a fragment thereof, and assaying the interaction between LXRα and the heterodimerization partner, preferably by time resolved fluorescence resonance energy transfer or by scintillation proximity assay

iv) measurement of LXRα transcription or translation in a cell line expressing LXRα.

v) measurement of direct compound binding or competitive binding to LXRα, preferably by time resolved fluorescence resonance energy transfer or scintillation proximity assay.

Examples of a suitable assays can be found in WO 99/18124 (EP1021462) Merck & Co.

Examples of suitable co-activators, but not limited to, are the Steroid Receptor Coactivators, such as SRC-1, SRC-2, and SRC-3, the Nuclear Receptor CoActivators, such as NcoA-1, NcoA-2, the CREB Binding protein (CBP), p300, p/CIP, TIF-1, TIF-2, TRIP-1, and GRIP-1.

Suitable heterodimerization partners are the Retinoid X Receptors (RXR), such as RXRα, RXRβ and RXRγ, preferably RXRα.

Preferably the cell line is a 3T3-L1 pre-adipocyte cell or a 3T3-L1 adipocyte cell or any other commanly used mammalian cell line.

The mammalian LXRα receptors may be conveniently isolated from commercially available RNA, brain cDNA libraries, genomic DNA, or genomic DNA libraries using conventional molecular biology techniques such as library screening and/or Polymerase Chain Reaction (PCR). These techniques are extensively detailed in Molecular Cloning – A Laboratory Manual, 2nd edition, Sambrook, Fritsch & Maniatis, Cold Spring Harbor Press.

The resulting cDNA's encoding mammalian LXRα receptors are then cloned into commercially available mammalian expression vectors such as the pcDNA3 series (InVitrogen Ltd etc. see below). An alternative mammalian expression vector is disclosed by Davies et al., J of Pharmacol & Toxicol. Methods, 33, 153-158. Standard transfection technologies are used to introduce these DNA's into commonly available cultured, mammalian cell lines such as CHO, HEK293, HeLa and clonal derivatives expressing the receptors are isolated. An alternative expression system is the MEL cell expression system claimed in our UK patent no. 2251622.

Application of a natural ligand to these cells causes activation of the transfected receptor that may cause changes in the levels of endogenous molecules such as ABC-1 or aFABP These may all be measured using standard published procedures and commercially available reagents. In addition, these cDNA's may be transfected into derivatives of these cells lines that have previously been transfected with a "reporter" gene. Examples of suitable reporter genes are esterase, phosphatases, proteases, fluorescent proteins, such as GFP, YFP,

BFP, and CFP, luciferase, chloramphenicol acetyl transferase, β-galactosidase, β-glucuronidase that will "report" these intracellular changes.

These transfected cell lines may be used to identify low molecular weight compounds that activate these receptors, these are defined as "agonists".

In addition or alternatively, the same assays can be used to identify low molecular weight compounds that antagonise the activation effect of a LXRα ligand, these are defined as "antagonists". Antagonist may have utility in treating obesity, dyslipidemia, insulin resistance syndrome and type 2 diabetes.

The test compound may be a polypeptide of equal to or greater than, 2 amino acids such as up to 6 amino acids, up to 10 or 12 amino acids, up to 20 amino acids or greater than 20 amino acids such as up to 50 amino acids. For drug screening purposes, preferred compounds are chemical compounds of low molecular weight and potential therapeutic agents. They are for example of less than about 1000 Daltons, such as less than 800, 600 or 400 Daltons in weight. If desired the test compound may be a member of a chemical library. This may comprise any convenient number of individual members, for example tens to hundreds to thousands to millions etc., of suitable compounds, for example peptides, peptoids and other oligomeric compounds (cyclic or linear), and template-based smaller molecules, for example benzodiazepines, hydantoins, biaryls, carbocyclic and polycyclic compounds (eg. naphthalenes, phenothiazines, acridines, steroids etc.), carbohydrate and amino acids derivatives, dihydropyridines, benzhydryls and heterocycles (eg. triazines, indoles, thiazolidines etc.). The numbers quoted and the types of compounds listed are illustrative, but not limiting. Preferred chemical libraries comprise chemical compounds of low molecular weight and potential therapeutic agents.

In a further aspect of the invention we provide the use of a modulator of LXRα receptor activity or expression as an agent able to stimulate pre-adipocyte differentiation and thereby modify or ameliorate insulin resistance syndrome or dyslipidemia or type 2 diabetes.

In a further aspect of the present invention we provide a method of treating insulin resistance syndrome, dyslipidemia or type 2 diabetes which method comprises administering to a patient suffering such a disease a pharmaceutically effective amount of an agent, preferably identified using one or more of the methods of this invention, able to stimulate pre-adipocyte differentiation by modulating LXRα activity or expression and thereby modify or ameliorate the insulin resistance syndrome, dyslipidaemia or type 2 diabetes disease.

This invention further provides use of an agent able to stimulate pre-adipocyte differentiation by modulating LXRα activity in preparation of a medicament for the treatment of dyslipidemia or IRS or type 2 diabetes. Preferably the compound is an LXRα agonist.

According to another aspect of the present invention there is provided a method of preparing a pharmaceutical composition which comprises:

i) identifying an agent as useful for stimulation of pre-adipocyte differentiation according to a method as described herein; and

ii) mixing the agent or a pharmaceutically acceptable salt thereof with a pharmaceutically acceptable excipient or diluent.

It will be appreciated that the present invention includes the use of orthologues and homologues of the human LXRα receptor.

The degree of pre-adipocyte differentiation required for the treatment of the type 2 diabetes, IRS or dyslipidemia can be almost any level of stimulation over basal levels as measured in the patient suffering from the particular disease, preferably at least 10% increase in rate over basal levels. Preferably a compound should be administered which has an affinity (K_m) for LXRα below 100μM preferably below 1μM, as measured against the isolated receptor.

The pharmaceutical composition can further comprise a PPARγ agonist, preferably a thiazolidinedione such as Darglitazone, Rosiglitazone, Pioglitazone, or Troglitazone.

By the term "orthologue" we mean the functionally equivalent receptor in other species.

By the term "homologue" we mean a substantially similar and/or related receptor in the same or a different species.

For either of the above definitions we believe the receptors may have for example at least 30%, such as at least 40%, at least 50%, at least 60%, and in particular at least 70%, such as at least 80%, for example 85%, or 90% or 95% peptide sequence identity. It is appreciated that homologous receptors may have substantially higher peptide sequence identity over small regions representing functional domains. We include receptors having greater diversity in their DNA coding sequences than outlined for the above amino acid sequences but which give rise to receptors having peptide sequence identity falling within the above sequence ranges. Convenient versions of the LXRα receptor include the published sequence. The amino acid sequence of human LXRα can be obtained from the SwissProt database, accession no Q13133 (NRH3_HUMAN) and the cDNA sequence e.g. from the EMBL database accession no.

U22662. The LXRα receptor is from any mammalian species, including human, monkey, rat, mouse and dog. Preferably the human LXRα receptor is used.

Fragments and partial sequences of the LXRα receptor may be useful substrates in the assay and analytical methods of the invention. It will be appreciated that the only limitation on these is practical, they must comprise the necessary functional elements for use in the relevant assay and/or analytical procedures.

The agent of this invention may be administered in standard manner for the condition that it is desired to treat, for example by oral, topical, parenteral, buccal, nasal, or rectal administration or by inhalation. For these purposes the compounds of this invention may be formulated by means known in the art into the form of, for example, tablets, capsules, aqueous or oily solutions, suspensions, emulsions, creams, ointments, gels, nasal sprays, suppositories, finely divided powders or aerosols for inhalation, and for parenteral use (including intravenous, intramuscular or infusion) sterile aqueous or oily solutions or suspensions or sterile emulsions.

Knowledge of the LXRα receptor also provides the ability to regulate its expression *in vivo* by for example the use of antisense DNA or RNA. Thus, according to a further aspect of the invention we provide an appetite control agent comprising an antisense DNA or an antisense RNA which is complementary to all or a part of a polynucleotide sequences shown in sequence nos. 1,3 and 5. By complementary we mean that the two molecules can hybridise to form a double stranded molecule through nucleotide base pair interactions to the exclusion of other molecular interactions.

The antisense DNA or RNA for co-operation with polynucleotide sequence corresponding to all or a part of a LXRα gene can be produced using conventional means, by standard molecular biology and/or by chemical synthesis. The antisense DNA or RNA can be complementary to the full length LXRα receptor gene of the invention or to a fragment thereof. Antisense molecules which comprise oligomers in the range from about 12 to about 30 nucleotides which are complementary to the regions of the gene which are proximal to, or include, the protein coding region, or a portion thereof, are preferred embodiments of the invention. If desired, the antisense DNA or antisense RNA may be chemically modified so as to prevent degradation *in vivo* or to facilitate passage through a cell membrane and/or a substance capable of inactivating mRNA, for example ribozyme, may be linked thereto and the invention extends to such constructs.

Oligonucleotides which comprise sequences complementary to and hybridizable to the

LXRα receptor are contemplated for therapeutic use. U.S. Patent No. 5,639,595, *Identification of Novel Drugs and Reagents*, issued Jun. 17, 1997, wherein methods of identifying oligonucleotide sequences that display *in vivo* activity are thoroughly described, is herein incorporated by reference.

Nucleotide sequences that are complementary to the LXRα receptor encoding nucleic acid sequence can be synthesised for antisense therapy. These antisense molecules may be DNA, stable derivatives of DNA such as phosphorothioates or methylphosphonates, RNA, stable derivatives of RNA such as 2'-O-alkylRNA, or other oligonucleotide mimetics. U.S. Patent No. 5,652,355, *Hybrid Oligonucleotide Phosphorothioates*, issued July 29, 1997, and U.S. Patent No. 5,652,356, *Inverted Chimeric and Hybrid Oligonucleotides*, issued July 29, 1997, which describe the synthesis and effect of physiologically-stable antisense molecules, are incorporated by reference. LXRα gene antisense molecules may be introduced into cells by microinjection, liposome encapsulation or by expression from vectors harbouring the antisense sequence.

Transgenic animal technology is also contemplated, providing new experimental models, useful for evaluating the effects of test compounds on the control of dyslipidemia, insulin resistance syndrome, type 2 diabetes, obesity and eating disorders. LXRα may be deleted, inactivated or modified using standard procedures as outlined briefly below and as described for example in "Gene Targeting; A Practical Approach", IRL Press, 1993. The target gene or a portion of it, for example homologous sequences flanking the coding region, is preferably cloned into a vector with a selection marker (such as Neo) inserted into the gene to disrupt its function. The vector is linearised then transformed (usually by electroporation) into embryonic stem cells (ES) cells (eg derived from a 129/Ola strain of mouse) and thereafter homologous recombination events take place in a proportion of the stem cells. The stem cells containing the gene disruption are expanded and injected into a blastocyst (such as for example from a C57BL/6J mouse) and implanted into a foster mother for development. Chimaeric offspring may be identified by coat colour markers. Chimeras are bred to ascertain the contribution of the ES cells to the germ line by mating to mice with genetic markers which allow a distinction to be made between ES derived and host blastocyst derived gametes. Half of the ES cell derived gametes will carry the gene modification. Offspring are screened (for example by Southern blotting) to identify those with a gene disruption (about 50% of the progeny). These selected offspring will be heterozygous and may therefore be bred with another heterozygote to produce homozygous offspring (about 25% of the progeny).

Transgenic animals with a target gene deletion ("knockouts") may be crossed with transgenic animals produced by known techniques such as microinjection of DNA into pronuclei, spheroplast fusion or lipid mediated transfection of ES cells to yield transgenic animals with an endogenous gene knockout and a foreign gene replacement. ES cells containing a targeted gene disruption may be further modified by transforming with the target gene sequence containing a specific alteration. Following homologous recombination the altered gene is introduced into the genome. These embryonic stem cells may subsequently be used to create transgenics as described above. Suitable methods are described in WO 00/34461 University of Texas.

The transgenic animals will display a phenotype, which reflects the role of LXRα in the control of appetite and obesity and will thus provide useful experimental models in which to evaluate the effects of test compounds. Therefore in a further aspect of the invention we provide transgenic animals in which LXRα is deleted, inactivated or modified, and used in evaluating the effects of test compounds in dyslipidemia, insulin resistance syndrome, type 2 diabetes, appetite control and obesity. The LXRα receptor may also be used as the basis for diagnosis, for example to determine expression levels in a human subject, by for example direct DNA sequence comparison or DNA/RNA hybridisation assays. Diagnostic assays may involve the use of nucleic acid amplification technology such as PCR and in particular the Amplification Refractory Mutation System (ARMS) as claimed in our European Patent No. 0 332 435. Such assays may be used to determine allelic variants of the gene, for example insertions, deletions and/or mutations such as one or more point mutations. Such variants may be heterozygous or homozygous. Other approaches have been used to identify mutations in genes encoding similar molecules in obese patients (Yeo et al., 1998, Nature Genetics, 20, 111-112).

In a further aspect of the invention the LXRα receptor can be genetically engineered in such a way that its interactions with other intracellular and membrane associated proteins are maintained but its effector function and biological activity are removed. The genetically modified protein is known as a dominant negative mutant. Overexpression of the dominant negative mutant in an appropriate cell type down regulates the effect of the endogenous protein, thus revealing the biological role of the genes in dyslipidemia, insulin resistance syndrome, type 2 diabetes.

Similarly, the LXRα receptor may also be genetically engineered in such a way that its effector function and biological activity are enhanced. The resultant overactive

protein is known as dominant positive mutant. Overexpression of a dominant positive mutant in an appropriate cell type amplifies the biological response of the endogenous, native protein, spotlighting its role in dyslipidemia, insulin resistance syndrome, type 2 diabetes. This also has utility in a screen for detecting anatgonists of the constitutively active receptor in the absence of a ligand.

Therefore, in a further aspect of the invention we provide dominant negative and dominant positive mutants of a LXRα receptor and their use in evaluating the biological role of the LXRα receptor in the control of insulin resistance syndrome, dyslipidemia or type 2 diabetes.

The invention will now be illustrated but not limited by reference to the following specific description and sequence listing [Many of the specific techniques used are detailed in standard molecular biology textbooks such as Sambrook, Fritsch & Maniatis, Molecular cloning, a Laboratory Manual, Second Edition, 1989, Cold Spring Harbor Laboratory Press. Consequently references to this will be made at the appropriate points in the text.]:

EXAMPLES

The effect of PPARγ activators on LXRa expression in 3T3-L1 adipocytes

We performed Northern blot analysis on total RNA from 3T3-L1 adipocytes treated with increasing doses of a PPARγ agonist. Adipocytes treated over a 24 hrs period with 0.01, 0.1, and 1 mM of Darglitazone. 20 μg total RNA was subjected to Northern blotting and probed with a ^{32}P-labeled LXRα cDNA probe. The signal was obtained by scanning the autoradiogram and normalised for 18S RNA expression. Results showed an approximately 5-fold induction of LXRa mRNA (Figure 1). These concentrations are in agreement with concentrations required for activation of PPARγ in reporter assays (Lehmann et al. 1995). Hence, treatment of adipocytes with a selective PPARγ agonist increases LXRα mRNA levels.

Treatment of 3T3-L1 adipocytes with 22-R-hydroxy cholesterol and Darglitazone

3T3-L1 cells committed to adipocyte differentiation were treated with either Darglitazone, the LXRα agonist 22-R-hydroxy cholesterol, or both. 22-R-hydroxy cholesterol (22-R) is a naturally occurring agonist for LXRα (Janowski et al. 1996). Cells stimulated with 22-R, Darglitazone or both were forming gradually larger lipid droplets, as shown by Oil Red-O staining of the cells. These results indicate that Darglitazone stimulation of PPARγ as well

as 22-R stimulation of LXRα leads to increased storage of triglycerides in adipocytes. In parallel, Northern blot analysis of total RNA shows an increase of LXRα mRNA in differentiating 3T3-L1 cells treated with Darglitazone or 22-R and an additive effect by stimulation with both Darglitazone and 22-R (Figure 2). Therefore, treatment of 3T3-L1 pre-adipocytes with either a PPARγ agonist or an LXRα agonist leads to fat accumulation and increased expression of LXRα.

Treatment of human adipocytes with 22-R-hydroxy cholesterol

Human adipocytes were obtained from breast reduction surgery. Pieces of adipose tissue (5-600 mg) were prepared under sterile conditions and used for incubations in plastic tubes essentially as described (Ottosson et al., 1994). 1 μM Darglitazone, 5 μM 22-(R)-hydroxy cholesterol or both was added for 48 hrs as indicated in the figure legends. PolyA+ RNA was isolated and subjected to Northern blot analysis (Figure 3). Both Darglitazone and 22-(R)-hydroxy cholesterol treatment led to increased expression levels of LXRα mRNA with an additive effect. Hence, stimulation with a selective PPARγ agonist or an LXRa agonist leads to upregulation of LXRα mRNA in human adipocytes.

Cells and reagent

The 3T3-L1 cell line (ATCC) was maintained in DMEM supplemented with 10 % fetal calf serum, 2 mM L-glutamine and penicillin/streptomycin at 37 °C. Cells were grown to confluence and exposed to adipogenic reagents for 3 days, followed by culturing for 3 more days in medium containing insulin only as described elsewhere (Lin and Lane, 1992). Insulin was used at a concentration of 1 μg/ml, dexamethasone at 1 μM and isobutylmethylxanthine at 0.5 mM.

Preparation and analysis of RNA

Total RNA from differentiated 3T3-L1 adipocytes or adipose tissue were extracted by the Trizol (Life Technologies, Inc.) method as recommended by the manufacturer. Northern blot analysis of RNA was performed as described earlier (Sorensen et al., 1994). 20 μg of total RNA was analyzed for LXRα and ribosomal protein 18S mRNA.

Oil Red O staining

Light microscopy and Oil Red O staining were used to monitor the characteristic cell rounding and lipid droplet accumulation in these cells during differentiation. Images were taken using a microscope (Leica DMIL) and a dual-colour charge coupled device camera (Leica MPS 60).

Claims:

1. A method of stimulating pre-adipocyte differentiation in a cell comprising administering a LXRα agonist to the cell, wherein the agonist stimulates pre-adipocyte differentiation.

2. The method of claim 1, wherein the cell is a mammalian cell.

3. The method of claim 1, wherein the cell is an adipocyte cell, a 3T3-L1 pre-adipocyte cell, or a 3T3-L1 adipocyte cell.

4. The method of claim 1, wherein the LXRα agonist is an oxidized derivative of cholesterol.

5. The method of claim 4, wherein the derivative is selected from the group consisting of 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol.

6. The method of claim 1, wherein the LXRα agonist is a thiazolidinedione compound.

7. The method of claim 6, wherein the thiazolidinedione compound is selected from the group consisting of darglitazone, rosiglitazone, pioglitazone, or troglitazone, and their pharmaceutically acceptable salts.

8. A method of treating a disorder associated with aberrant pre-adipocyte differentiation, comprising administering a therapeutically effective amount of a LXRα modulator to a mammal, wherein the LXRα modulator stimulates pre-adipocyte differentiation.

9. The method of claim 8, wherein the LXRα modulator is an oxidized derivative of cholesterol.

10. The method of claim 9, wherein the derivative is selected from the group consisting of 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol.

11. The method of claim 8, wherein the LXRα modulator is a thiazolidinedione compound.

12. The method of claim 11, wherein the thiazolidinedione compound is selected from the group consisting of darglitazone, rosiglitazone, pioglitazone, or troglitazone, and their pharmaceutically acceptable salts.

13. The method of claim 8, wherein the disorder is insulin resistance syndrome, dyslipidemia or type 2 diabetes.

14. The method of claim 8, wherein the LXRα modulator is administered to the mammal in a pharmaceutical composition comprising a pharmaceutically acceptable carrier or excipient.

15. A method of increasing the level of LXRα expression or activity in a pre-adipocyte cell, comprising administering a pharmaceutically effective amount of a LXRα modulator.

16. The method of claim 15, wherein the LXRα modulator is an oxidized derivative of cholesterol.

17. The method of claim 16, wherein the derivative is selected from the group consisting of 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol.

18. The method of claim 15, wherein the LXRα modulator is a thiazolidinedione compound.

19. The method of claim 18, wherein the thiazolidinedione compound is selected from the group consisting darglitazone, rosiglitazone, pioglitazone, or troglitazone, and their pharmaceutically acceptable salts.

20. The method of claim 15, wherein the modulator is administered to a mammal.

21. The method of claim 20, wherein the mammal has insulin resistance syndrome, dyslipidemia or type 2 diabetes.

22. A method for identifying a compound that stimulates pre-adipocyte differentiation, the method comprising:

providing a pre-adipocyte cell or a adipocyte cell comprising a LXRα regulatory

sequence operatively linked to a reporter gene;

introducing a test compound into the cell; and

assaying for transcription of the reporter gene in the cell, wherein an increase in transcription in the presence of the compound compared to transcription in the absence of the compound indicates that the compound stimulates pre-adipocyte differentiation in the cell.

23. The method of claim 22, wherein the cell is a mammalian cell.

24. The method of claim 23, wherein the cell is a 3T3-L1 pre-adipocyte cell, or a 3T3-L1 adipocyte cell.

25. The method of claim 22, wherein the reporter gene encodes a luciferase, a chloramphenicol acetyl transferase, a beta-galactosidase, an alkaline phosphate, or a fluorescent protein.

26. A method of identifying an agonist of LXRα comprising:

contacting a LXRα protein, or fragment thereof, a LXRα coactivator and a compound; and

determining if the LXRα protein, or fragment thereof, and the LXRα coactivator interact, wherein an interaction between the LXRα protein, or fragment thereof, and the LXRα coactivator indicates that the compound is a LXRα agonist.

27. The method of claim 26, wherein the LXRα co-activator is a steroid receptor co-activator.

28. A method of identifying an agonist of LXRα comprising:

contacting a LXRα protein, or fragment thereof, a LXRα heterodimerization partner or fragment thereof, and a compound; and

determining if the LXRα protein, or fragment thereof, and the LXRα heterodimerization partner, or fragment thereof, interact, wherein an interaction between the LXRα protein, or fragment thereof, and the LXRα heterodimerization partner, or fragment thereof, indicates that the compound is a LXRα agonist.

29. The method of claim 28, wherein the LXRα heterodimerization partner is a retinoid X receptor.

30. Use of a LXRα modulator in the manufacture of a medicament for the treatment of a disorder associated with aberrant pre-adipocyte differentiation, wherein the LXRα modulator stimulates pre-adipocyte differentiation.

31. Use according to claim 30, wherein the LXRα modulator is an oxidized derivative of cholesterol.

32. Use according to claim 31, wherein the derivative is selected from the group of 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol.

33. Use according to claim 30, wherein the LXRα modulator is a thiazolidinedione compound.

34. Use according to claim 33, wherein the thiazolidinedione compound is selected from the group consisting of darglitazone, rosiglitazone, pioglitazone, or troglitazone, and their pharmaceutically acceptable salts.

35. Use according to claim 30, wherein the disorder is insulin resistance syndrome, dyslipidemia or type 2 diabetes.

36. Use according to claim 30, wherein the LXRα modulator is administered orally, topically, intravenously, transdermally, rectally, or parentally.

37. A pharmaceutical formulation for the use in the treatment of a disorder associated with aberrant pre-adipocyte differentiation.

38. A pharmaceutical formulation of claim 37, wherein the LXRα modulator is an oxidized derivative of cholesterol.

39. A pharmaceutical formulation of claim 38, wherein the derivative is selected from the

group of 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24,25(S)-epoxycholesterol.

40. A pharmaceutical formulation of claim 37, wherein the LXRα modulator is a thiazolidinedione compound.

41. A pharmaceutical formulation of claim 40, wherein the thiazolidinedione compound is selected from the group consisting of darglitazone, rosiglitazone, pioglitazone, or troglitazone, and their pharmaceutically acceptable salts.

42. A pharmaceutical formulation of claim 37, wherein the disorder is insulin resistance syndrome, dyslipidemia or type 2 diabetes.

43. A pharmaceutical formulation of claim 37, wherein the LXRα modulator is administered orally, topically, intravenously, transdermally, rectally, or parentally.

FIGURE 1

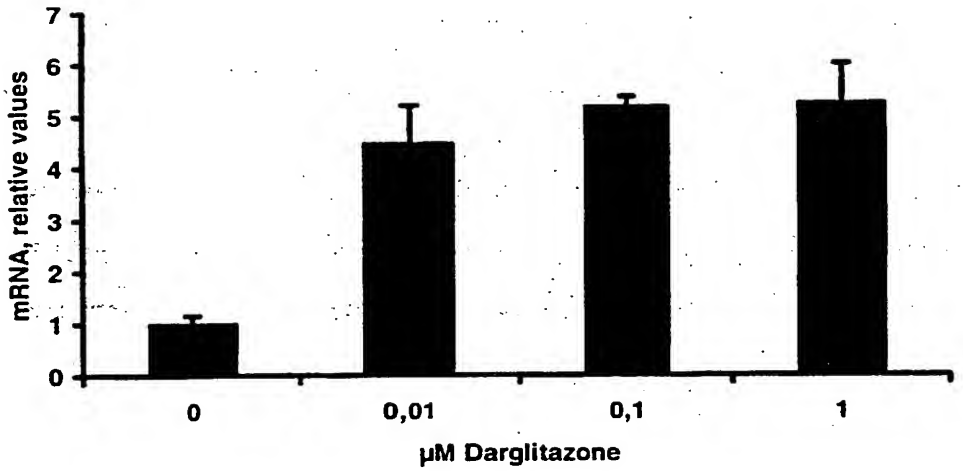


FIGURE 2

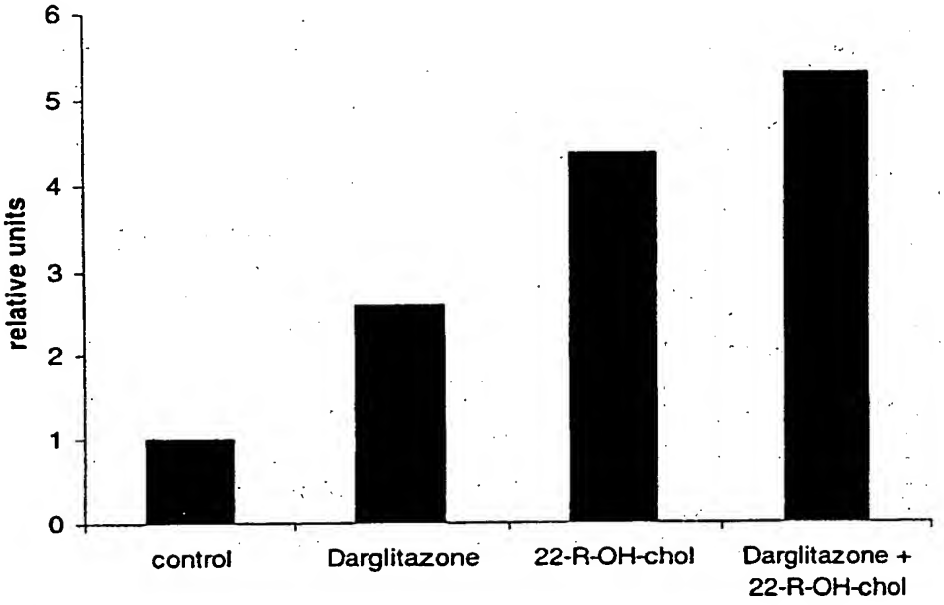
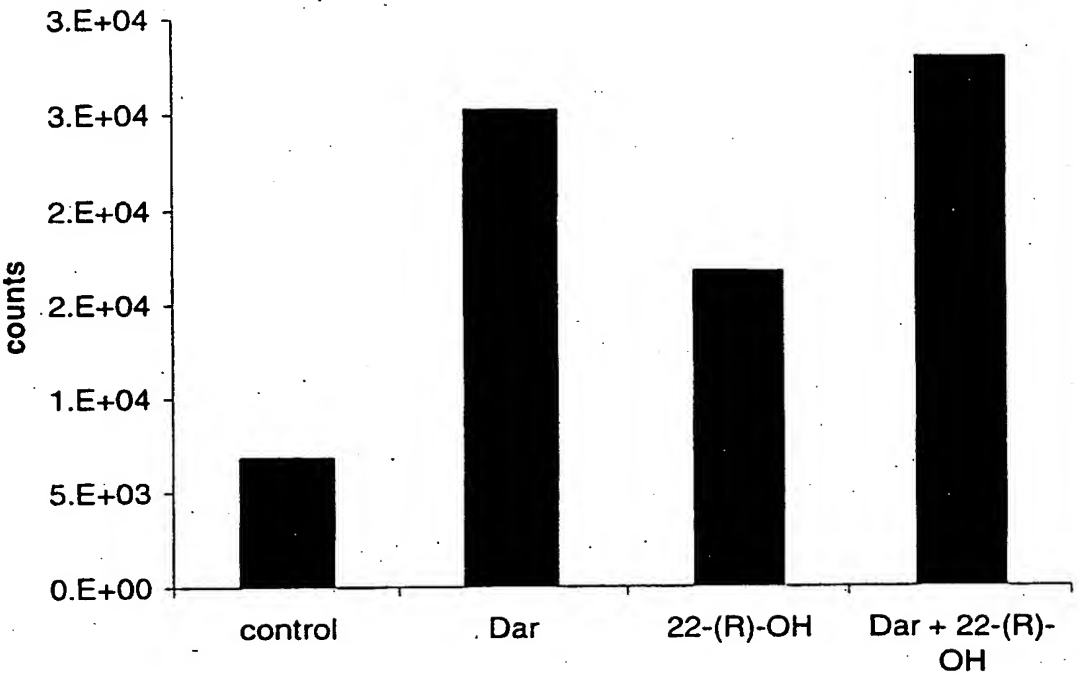

6
5
4
3
2
1
0
relative units
control
Darglitazone
22-R-OH-chol
Darglitazone +
22-R-OH-chol

FIGURE 3



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(74) Agent: **GLOBAL INTELLECTUAL PROPERTY**; AstraZeneca AB, S-151 85 Södertälje (SE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

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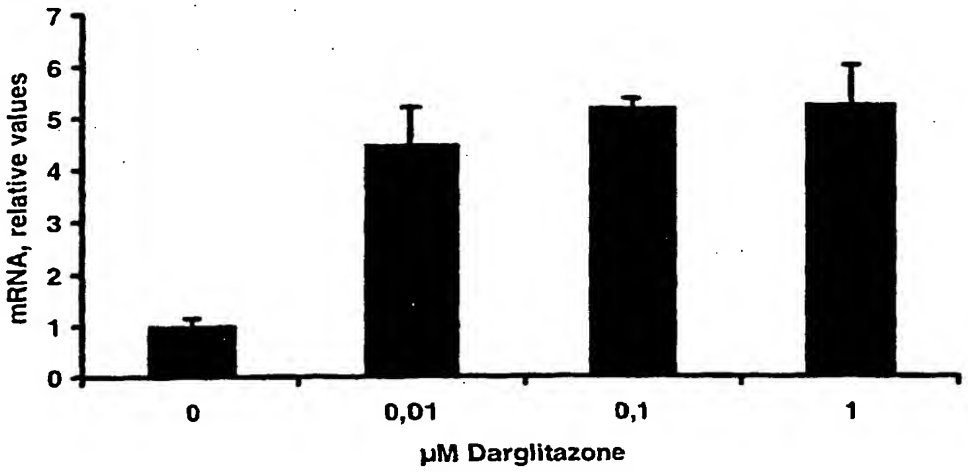
Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *of inventorship (Rule 4.17(iv)) for US only*

[Continued on next page]

(54) Title: METHOD FOR IDENTIFYING LIGANDS TO LXR-RECEPTOR THAT STIMULATES PRE-ADIPOCYTE DIFFERENTIATION AND FORMULATIONS THEREOF



(57) Abstract: The invention also relates to the use of active modulators of LXRα activity or expression in stimulation of pre-adipocyte differentiation and hence also in the treatment of insulin resistance syndrome, or dyslipidemia, or type 2 diabetes.

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Published:
— *with international search report*

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21 November 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 02/00102

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07D 277/34, C07K 14/705, A61P 3/10
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07D, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Nature, Volume 383, October 1996, Bethany A. Janowski et al: "An oxysterol signalling pathway mediated by the nuclear receptor LXRalpha", page 728 - page 731 --	28-29
X	Current Opinion in Genetics & Development, Volume 8, 1998, Daniel J Peet et al: "The LXRs:a new class of oxysterol receptors", page 571 - page 575 --	28-29
X	Proc. Natl. Acad. Sci., Volume 96, January 1999, Bethany A. Janowski et al: "Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta", page 266 - page 271 --	22-25,30-43

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 July 2002	16 -07- 2002
Name and mailing address of the ISA: Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Fernando Farieta/BS Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 02/00102

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Diabetes, Volume 46, August 1997, Didier Auboeuf et al: "Tissue Distribution and Quantification of the Expression of mRNAs of Peroxisome Proliferator-Activated Receptors and Liver X Receptor-alpha in Humans", page 1319 - page 1327	22-25,30-43
X	WO 0182917 A2 (TULARIK INC.), 8 November 2001 (08.11.01), claims 1-41, (specially claim 18)	22-25,30-43
P,X	WO 0117994 A1 (GLAXO GROUP LIMITED), 15 March 2001 (15.03.01), claims 1-15	22-25,30-43
X	WO 0066611 A1 (ARCH DEVELOPMENT CORPORATION), 9 November 2000 (09.11.00), page 34, line 13-24, claims 1-68	22-25,30-43
X	WO 9710813 A1 (LIGAND PHARMACEUTICALS INCORPORATED), 27 March 1997 (27.03.97), page 4, lines 2-30, claims 5, 8	22-25,30-43
A	WO 0034461 A2 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM), 15 June 2000 (15.06.00), claims 1-14, 21-29, 44-45	28-29
E,A	National Library of Medicine, (NLM), file Medline, Medline accession no. 21929611, Laffitte BA et al: "Orphan nuclear receptors find a home in the arterial wall"; & Curr Atheroscler Rep 2002 May;4(3):213-21, abstract	22-25,30-43
A	Ann. Endocrinol, Volume 62, no. 3, 2001, J.-M.A. Lobaccaro et al: "Régulation du métabolisme des lipides par les récepteurs nucléaires orphelins", page 239 - page 247, figure 3	28-29

INTERNATIONAL SEARCH REPORT

Inten l application No.
PCT/SE02/00102

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-21
because they relate to subject matter not required to be searched by this Authority, namely:

see next sheet*

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet**

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.: 22-25, 28-29,30-43

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

*
Claims 1-21 relate to methods of treatment of the human or animal body by surgery or by therapy/ diagnostic methods practised on the human or animal body/Rule 39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.

**

Regulation of lipid metabolism by the orphan nuclear receptors is well known in the art. The technical feature characteristic of invention 1 is a method for identifying a compound that stimulates pre-adipocyte differentiation. According to WO 97/10813 treating obesity, providing a pre-adipocyte or adipocyte cell linked to the activity of a receptor are known features in the determination of ligand variants. Furthermore WO 00/34461 present compositions and methods of modulating cholesterol metabolism. The mechanism to achieve such a modulation is through LXR- variants.
Claims 1-21 relate to methods of treatment of the human or animal body, by surgery or by therapy. (Claims 1-21 relate to 3 group of inventions namely: 1-6, 8-14 and 15-21). The search of claims 1-21 has not been performed according to PCT Rule 39.1(iv).

According to Article 34 (3)(a-c) and PCT Rule 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features", i. e. features that define a contribution which each of the inventions, makes over the prior art. From the rather broad wording of claims 1-21, it is not possible to retrieve such a common technical feature. These claims are considered to involve a multitude of features, many of which are revealed in the following subdivisions of claims 22-43. The present application is thus considered to relate to at least three groups of inventions, namely:

1. A method for identifying compounds that stimulates pre-adipocyte differentiation, using these as LXRα modulators and manufacture a pharmaceutical formulation thereof, according to claims 22-25 and 30-43.
2. A method of identifying an agonist of LXRα, contacting a LXRα-protein to a LXRα-co-activator and a compound, according to claims 26-27.
3. A method of identifying an agonist of LXRα, contacting a LXRα-protein to a LXRα-heterodimer and a compound, according to claims 28-29.

Invention 1 and 3 have been partially searched, to the extent it was possible considering the broad wording in claims 22, 30 and 37. The general aspects of the method have been covered in the search. The partial search of invention 3 covers mostly the named heterodimers from the description on page 8 lines10-11 (RXR).

Invention 2 concerns to co-activators of LXR as for example Steroid Receptor Co-activators (SRC) and other possibilities as named on page 8 lines 6-10. Invention 2 has not been searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 02/00102

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0182917 A2	08/11/01	AU 6298401 A	12/11/01
		US 2002048572 A	25/04/02
WO 0117994 A1	15/03/01	AU 7115300 A	10/04/01
		EP 1210345 A	05/06/02
WO 0066611 A1	09/11/00	AU 4667200 A	17/11/00
		EP 1189922 A	27/03/02
		NO 20015314 A	27/12/01
WO 9710813 A1	27/03/97	AU 725998 B	26/10/00
		AU 7074296 A	09/04/97
		AU 7074496 A	09/04/97
		BR 9610624 A	16/03/99
		CA 2204616 A	27/03/97
		CA 2232288 A	27/03/97
		EP 0788353 A	13/08/97
		EP 0859608 A	26/08/98
		JP 11511472 T	05/10/99
		NO 981192 A	18/05/98
		US 5972881 A	26/10/99
		US 6028052 A	22/02/00
		US 6228862 B	08/05/01
		US 6316404 B	13/11/01
		WO 9710819 A	27/03/97
WO 0034461 A2	15/06/00	AU 2051600 A	26/06/00

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Induction of the Nuclear Orphan Receptor RORγ during Adipocyte Differentiation of D1 and 3T3-L1 Cells

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Abstract

Here, we analyzed the expression of the three members of the retinoid-like orphan receptor (ROR) nuclear receptor subfamily during adipocyte differentiation. RORα and RORγ mRNA were up-regulated during adipocyte differentiation in preadipocyte D1 and 3T3-L1 cells, whereas RORβ mRNA could not be detected. The induction of RORα and RORγ mRNA succeeded the induction of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α and occurred at a similar time interval as did the increase in aP2 and lipoprotein lipase mRNA. Like the expression of PPARγ and aP2, the induction of RORγ mRNA was repressed by tumor necrosis factor α and transforming growth factor β. The induction of adipogenesis by prostaglandin D2 and two thiazolidinediones in the multipotent stem cells C3H10T1/2 was also accompanied by an induction in RORγ mRNA. In contrast to parental cells, clofibrate induces adipogenesis and RORα and RORγ mRNA in BALB/c3T3 cells that ectopically express PPARγ. RORγ mediates its effect on transcription through specific response elements. Cotransfection of RORα or RORγ and (RORγ response element)$_4$-chloramphenicol acetyltransferase into preadipocyte D1 cells induced transactivation of chloramphenicol acetyltransferase about 100-fold, suggesting that ROR plays a role in the regulation of gene expression in adipocytes. The nuclear orphan receptor Rev-ErbAα, which did not exhibit transactivation function, was able to inhibit transactivation by RORγ at two different levels. Our results show that RORγ is induced during adipocyte differentiation in D1 and 3T3-L1 cells and functions as an active transcription factor, suggesting a role for RORγ in the regulation of gene expression during this differentiation process.

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Introduction

Adipocytes are specialized cells that play an important role in energy homeostasis in vertebrate organisms. White adipocytes efficiently synthesize and store triglycerides in times of caloric excess and can hydrolyze and release unesterified fatty acids during periods of nutritional deprivation (1, 2). A number of murine *in vitro* model cell systems have been developed to study the regulation of this pathway of differentiation (1). These studies have demonstrated that preadipocytes are stimulated to differentiate at confluence by insulin and dexamethasone and that this differentiation can be inhibited by TGF-β[2] (3, 4) and TNF-α (5, 6). Adipocyte differentiation is accompanied by the accumulation of fat droplets and alterations in the expression of many genes, including the fatty acid-binding protein aP2 (7), the serine protease adipsin (8), and several enzymes involved in triglyceride metabolism.

The alterations in gene expression during adipocyte differentiation are regulated by a variety of transcriptional factors belonging to different families. Several members of the nuclear receptor superfamily have been found to play an important regulatory role in the induction of adipocyte differentiation. This family includes the steroid hormone, thyroid hormone, retinoid receptors, and orphan receptors, for which the ligand has not yet been identified (9, 10). One of the members, PPARγ, was found to be highly expressed in adipocytes and induced early during adipocyte differentiation (11–13). Activators for PPARγ that have been recently identified include the antidiabetic drugs thiazolidinediones and 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (14–17). The crucial role of this receptor was demonstrated by studies showing that retroviral expression of PPARγ in BALB/c or Swiss 3T3 cells induces adipocyte differentiation after treatment with PPAR activators (12). The importance of PPARγ in gene regulation during adipocyte differentiation was further illustrated by the presence of its REs in the 5′-promoter flanking region of several adipocyte-specific genes (18, 19). The PPARγ-dependent transactivation is mediated through a heterodimeric complex of this protein with the nuclear retinoid receptor RXR, thereby demonstrating the involvement of retinoid receptors in the control of adipocyte differentiation (19, 20). Recently, Rev-ErbAα, another member of the nuclear receptor family, was found to be induced during adipocyte differentiation of 3T3-L1 cells; however, its role in this process has yet to be

[2] The abbreviations used are: TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; PPAR, peroxisome proliferator-activated receptor; PG, prostaglandin; RE, response element; RXR, retinoid X receptor; C/EBP, CCAAT/enhancer binding protein; LPL, lipoprotein lipase; ROR, retinoid-like orphan receptor; IBMX, 3-isobutyl-1-methyl xanthine; RT-PCR, reverse transcription-PCR; GPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; DBD, DNA-binding domain.

determined (21). Several other transcriptional factors, in particular, members of the C/EBP family, have been reported to be important regulators of adipocyte differentiation. Ectopic expression of C/EBPβ has been shown to initiate adipocyte differentiation in NIH3T3 cells (22). Moreover, expression of C/EBPα has been demonstrated to promote adipocyte differentiation (22–26) and to cooperate with the PPARγ receptor (12, 27). A member of the forkhead/HNF3 family has been reported to be increased during adipocyte differentiation and to be involved in the regulation of the LPL gene (28).

Recently, our laboratory has identified a novel nuclear orphan receptor, referred to as RORγ (RORC, Genome Database Nomenclature Committee; Ref. 29), which is a new member of the ROR (also named RZR) subfamily (30–32). Members of this family bind as monomers to REs that consist of the core motif AGGTCA preceded by an A+T-rich region (30, 32). Each of the ROR receptors exhibits a distinct tissue distribution. RORα and RORγ have been shown to be expressed in many different tissues, whereas the expression of RORβ appears to be restricted to the brain (29–32). To study their function, we examined the expression of RORs during differentiation of several *in vitro* cell systems.

Here, we analyze the expression of members of the ROR family and, in particular, of RORγ during adipocyte differentiation. We demonstrate that the expression of RORα and RORγ correlates very closely with adipocyte differentiation in two preadipocyte cell lines D1 and 3T3-L1. The induction of these RORs appears to succeed the induction of PPARγ, suggesting that they may play a role in modulating gene expression at later stages of adipocyte differentiation rather than in the induction of differentiation itself. The observed opposing activities of the RORs and Rev-ErbAα receptors support the concept of cross-talk and competition between overlapping receptor signaling networks during this pathway of differentiation.

Results

Expression of RORs during Adipogenesis. To study the regulation of the expression of RORα, RORβ, and RORγ during adipocyte differentiation, the level of mRNA expression was examined in logarithmic cultures of several fibroblast and preadipocyte cell lines, as well as in confluent cultures maintained under differentiation-inducing conditions (5 days at confluence in the presence of dexamethasone and insulin). Under these differentiation-inducing conditions, only cultures of 3T3-L1 and D1 cells underwent adipocyte differentiation, and about 95% of the D1 cells and 45% of the 3T3-L1 cells contained fat droplets. The additional presence of 0.5 mM IBMX further stimulated differentiation in 3T3-L1 cells and increased the number of fat-containing cells to 95%; however, the addition of IBMX had little effect on adipogenesis in D1 cells (data not shown). The D1 cells exhibited a faster rate of accumulation of fat droplets than did 3T3-L1 cells (data not shown). RORβ mRNA, which has been reported to be highly brain specific (32), was undetectable in all cell lines under both culture conditions (data not shown). BALB/c3T3 cells contained very low levels of RORα or RORγ mRNA, whereas Swiss 3T3 cells expressed RORα but not RORγ mRNA (Fig. 1). RORα was expressed at similar levels in Swiss 3T3 cells from the exponential growth phase and from cultures maintained at differentiation-inducing conditions. Both RORα and RORγ mRNA were undetectable in undifferentiated 3T3-L1 and D1 cells but were expressed in differentiated cells (Fig. 1*A*). In differentiated cells, RORα mRNA was expressed at about 10-fold lower levels than was RORγ mRNA. Similar results were obtained when 3T3-L1 cells were treated with dexamethasone and insulin in the presence of IBMX (Fig. 1*B*). These results indicate that, except for Swiss 3T3 cells, the pattern of RORα mRNA expression is associated with the adipocyte phenotype. In particular, the pattern of RORγ mRNA expression correlated very well with the expression of the adipocyte phenotype. This conclusion was supported by the observed correlation between the expression patterns of RORα and RORγ mRNA and those of several genes known to be induced during adipocyte differentiation. The relatively high induction of RORγ mRNA in differentiating 3T3-L1 and D1 cells correlated well with that of the nuclear receptor PPARγ, C/EBPα, and the fat cell differentiation marker aP2 (Fig. 1). The PPARγ mRNA was expressed about equally in D1 and 3T3-L1 cells, whereas the relative level of RORγ mRNA was significantly higher in D1 cells compared to 3T3-L1 cells. In mouse myoblast 3T3-C2-C12 cells, which can be induced to differentiate into myotubes, very low RORγ mRNA expression was observed in both differentiated and undifferentiated cells (data not shown).

Time Course of RORγ mRNA Induction during Adipogenesis. We next examined the time course of the induction of RORα and RORγ mRNA to determine whether the induction occurs at an early or a late stage in adipogenesis. D1 cells were grown to confluence and then treated with dexamethasone and insulin. Fig. 2*A* shows the increase in the percentage of fat cells with time of treatment. A sharp increase in the number of adipocytes was observed after 2 days of treatment. PPARγ and C/EBPα, which have been reported to be induced early during adipocyte differentiation (11, 26, 27), were induced in D1 preadipocytes 24 h after the addition of dexamethasone and insulin (Fig. 2*B*). This induction was followed by an enhancement in the level of aP2 and LPL mRNA. Similar findings have been reported for 3T3-L1 (12). A sharp increase in the expression of RORα and RORγ mRNA was observed at days 2 and 3, respectively, at a time interval very similar to that for the induction of aP2 and the increase in the number of fat cells (Fig. 2). The level of GPDH mRNA, which was used as a control, was minimally affected during adipogenesis. The induction of RORα and RORγ mRNA succeeded the increase in PPARγ and C/EBPα, suggesting that RORα and RORγ may modulate gene expression at a later stage of differentiation rather than play a role in the induction of adipocyte differentiation.

Effect of TNF-α and TGF-β1 on RORγ and RORγ Expression. Several cytokines, including TGF-β1 and TNF-α, have been shown to inhibit adipocyte differentiation in 3T3-L1 cells (3–6). Both TGF-β1 and TNF-α blocked adipocyte differentiation in D1 preadipocytes, as indicated by the lack of accumulation of fat droplets in the cells (data not shown). The block in adipocyte differentiation was confirmed by the lack of PPARγ and aP2 mRNA induction. As shown in

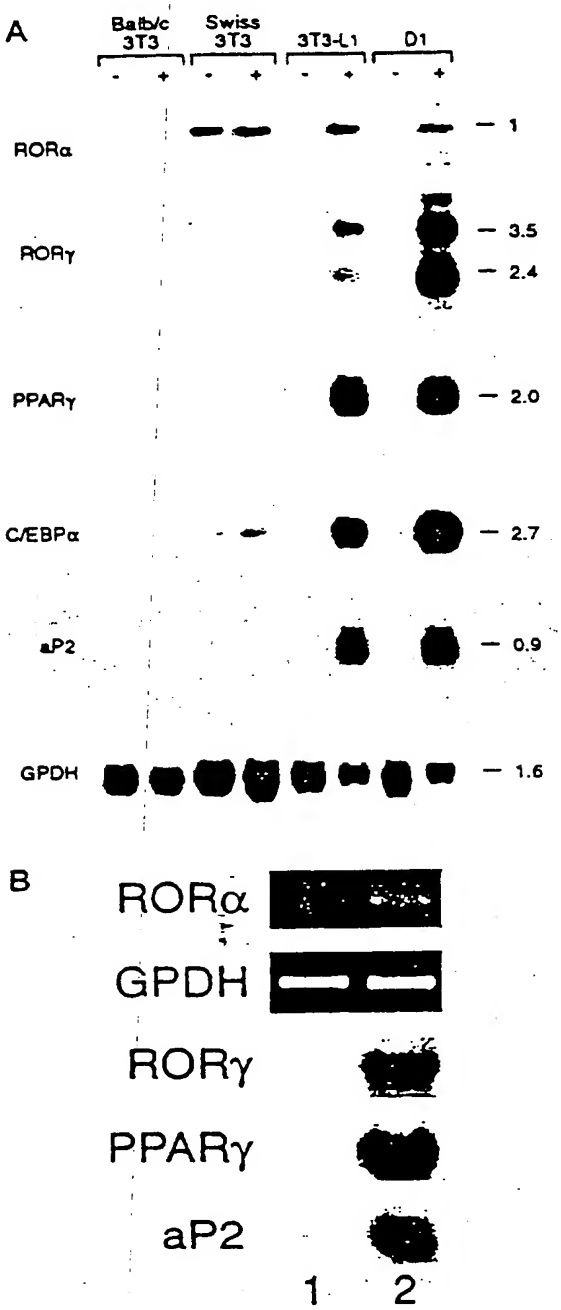

Fig. 1. *A*, differential expression of RORα and RORγ in several fibroblast and preadipocyte cell lines. Cells grown in the exponential phase (*Lanes −*) or maintained at confluence in the presence of dexamethasone and insulin for 5 days (*Lanes +*) were collected, and total RNA was isolated. At the time of collection, 0% of the BALB/c and Swiss 3T3 cells, 45% of the 3T3-L1, and 95% of the D1 cells contained fat droplets. *B*, 3T3-L1 cells were treated with insulin, dexamethasone, and 0.5 mM IBMX for 5 days, as described in "Materials and Methods." At the time of collection, 95% of the 3T3-L1 cells contained fat droplets. RNA (30 μg) was then examined by Northern blot analysis using radiolabeled probes for RORα, RORγ, PPARγ, C/EBPα, aP2, and GPDH. In *B*, the level of GPDH and RORα RNA was determined by RT-PCR because RORα is expressed as low levels and Northern analysis requires a 2-week exposure. The difference in levels of GPDH mRNA between undifferentiated and differentiated 3T3-L1 or D1 cells in *A* is due to unequal RNA loading. GPDH mRNA expression does not change significantly during adipocyte differentiation (see Fig. 2).

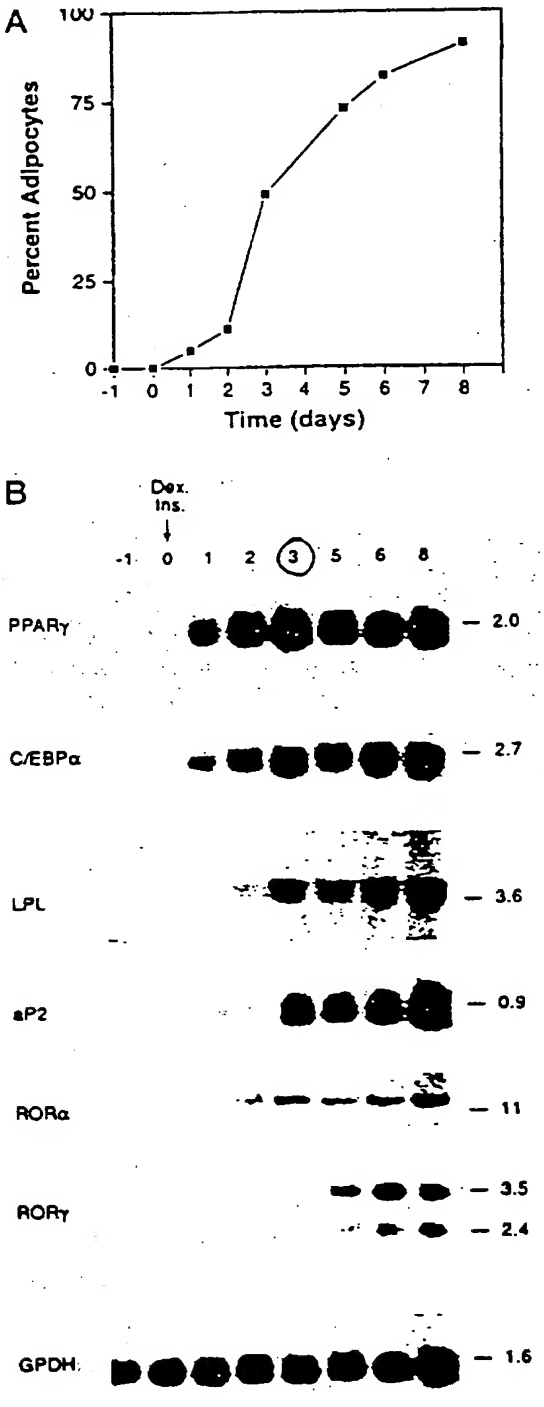

Fig. 2. Comparisons between the induction of RORα and RORγ mRNA, adipocyte differentiation, and the induction of adipocyte-specific genes. Preadipocyte D1 cells were grown to confluence and treated with dexamethasone and insulin (day 0). *A*, at the indicated times, cells were stained with Oil Red O, and the number of adipocytes was determined. In duplicate dishes, cells were collected, and total RNA was isolated. *B*, RNA was examined by Northern blot analysis using radiolabeled probes for RORα and RORγ. Blots were stripped and rehybridized with probes for PPARγ, C/EBPα, aP2, and LPL. A probe for GPDH was used as a control for equal loading of RNA.

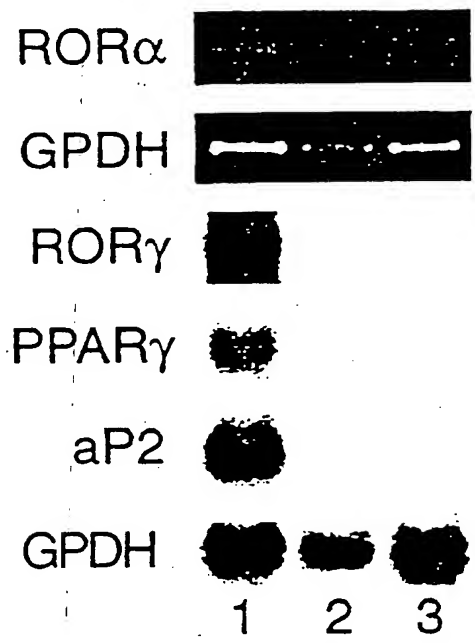

Fig. 3. Effect of TGF-β1 and TNF-α on the expression of RORα and RORγ mRNA. Preadipocyte D1 cells were grown to confluence, and then cultures were maintained in medium containing dexamethasone and insulin in the absence or presence of TNF-α (5 nM) or TGF-β1 (100 pM). After 5 days of treatment, cells were collected, and total RNA was isolated. RNA was examined by RT-PCR (*top two rows*) or Northern analysis using primers or probes for RORα and RORγ, PPARγ, aP2, and GPDH.

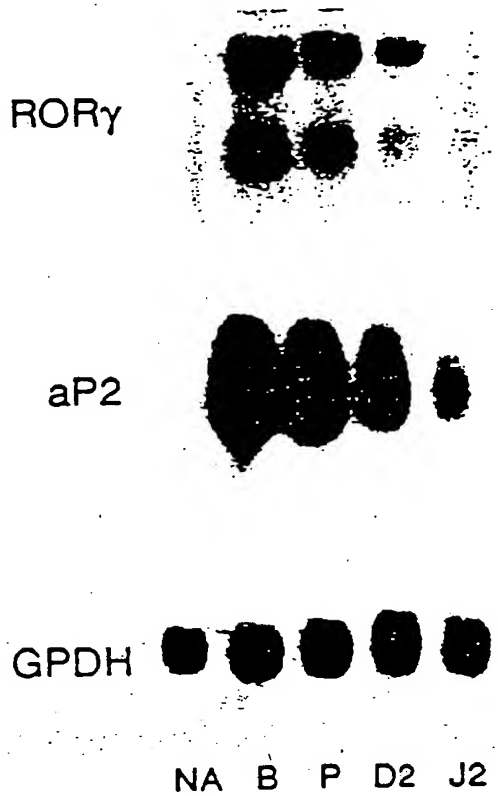

Fig. 4. Induction of RORγ mRNA by antidiabetic thiazolidinediones and prostaglandins in C3H10T1/2 cells. Confluent cultures were maintained in medium with dexamethasone and insulin in the absence (*Lane NA*) or presence of 1 μM each of BRL49653 (*Lane B*), pioglitazone (*Lane P*), PGD_2 (*Lane D2*), or 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (*Lane J2*). After 7 days, cells were collected, and total RNA was isolated. RNA was then examined by Northern analysis using radiolabeled probes for RORγ and aP2.

Fig. 3, both TGF-β1 and TNF-α suppressed the expression of RORα and RORγ mRNA (Fig. 3). These results lend further support for the conclusion that RORα and RORγ expression is closely associated with the expression of the adipocyte phenotype in these cells.

Induction of RORγ mRNA by Antidiabetic Drugs and Prostaglandins. Recently, it was discovered that several thiazolidinediones, such as BRL49653 and pioglitazone, which have been used as antidiabetic drugs, as well as 15-deoxy-$\Delta^{12,14}$-PGJ_2, can bind PPARγ and promote adipocyte differentiation in the multipotent stem cells C3H10T1/2 (15–17). We, therefore, examined whether these agents were able to induce the expression of RORγ in these cells. In agreement with previous findings (15), treatment of C3H10T1/2 cells with BRL49653 and pioglitazone induced accumulation of lipid (data not shown) and increased expression of aP2 (Fig. 4). This change in phenotype and gene expression was accompanied by an induction of RORγ mRNA expression (Fig. 4). A similar result was obtained when cells were treated with 15-deoxy-$\Delta^{12,14}$-PGJ_2 or PGD_2, which functions as a precursor of 15-deoxy-$\Delta^{12,14}$-PGJ_2 (Fig. 4). However, the induction of adipocyte differentiation and RORγ expression by 15-deoxy-$\Delta^{12,14}$-PGJ_2 was very weak, most likely because of its instability in aqueous solutions. BRL49653 was the most effective in increasing the level of RORγ mRNA, followed by pioglitazone, PGD_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2. RT-PCR could not detect any RORα mRNA in either treated or untreated C3H10T1/2 cells. These findings show that activation of PPARγ results in an increase in RORγ mRNA expression and further strengthen the hypothesis that induction of RORγ expression accompanies adipocyte differentiation in these cells.

Ectopic Expression of PPARγ in Balb/c3T3. Overexpression of PPARγ, followed by treatment with PPAR activators, has been demonstrated to cause induction of adipocyte differentiation in several 3T3 cell lines, which normally are unable to differentiate (12, 18). To analyze whether constitutive expression of PPARγ in BALB/c3T3 cells results in an induction of RORα and RORγ, we infected BALB/c3T3 cells with a retroviral vector expressing PPARγ or with the control vector pBABE. After selection by puromycin, resistant cells were grown to confluence and treated with clofibrate for 8 days. Cells were then examined for RORα and RORγ mRNA expression. Like parental cells, BALB/c3T3 cells transfected with the control vector pBABE were unable to undergo adipocyte differentiation in the presence of clofibrate, as indicated by the absence of fat droplets in the cells (data not shown) and the lack of detectable aP2 and PPARγ mRNA expression (Fig. 5). These cells also did not express RORα and RORγ mRNA. In contrast, clofibrate-treated cultures of BALB/c3T3-PPARγ cells also expressed, in addition

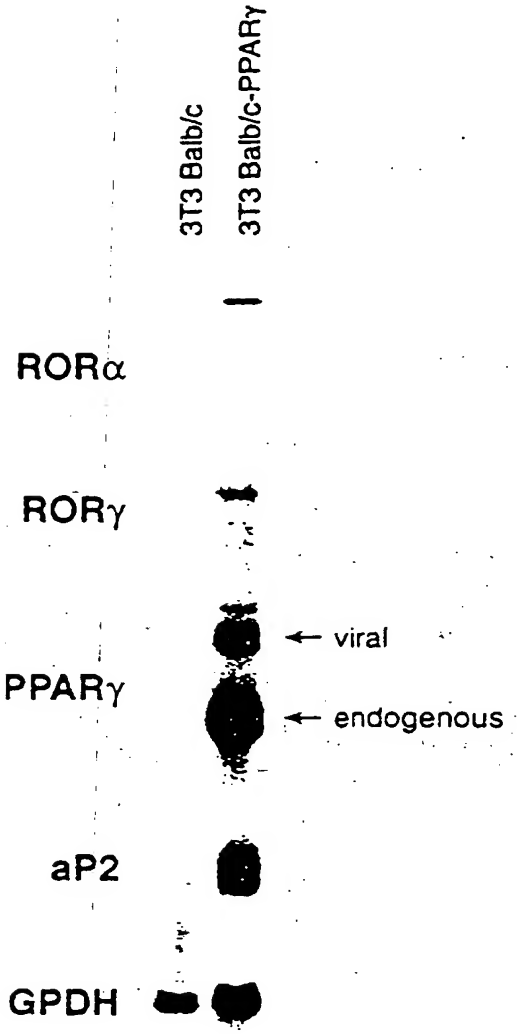

Fig. 5. Ectopic expression of PPARγ in BALB/c3T3 cells induces RORα and RORγ. BALB/c3T3 cells were infected with retroviruses carrying pBABE or pBABE-PPARγ expression vectors, as described in "Materials and Methods." Cells were grown to confluence and treated with dexamethasone and insulin in the presence of clofibrate (100 μM). After 5 days, total RNA was isolated and examined by Northern blot analysis for expression of PPARγ, aP2, RORα, RORγ, and GPDH mRNA. *Lane 3T3 BALB/c*, cells infected with pBABE; *3T3 BALB/c-PPARγ*, cells infected with pBABE-PPARγ. The transcripts of the endogenous and viral (transgene) PPARγ gene are indicated at the right.

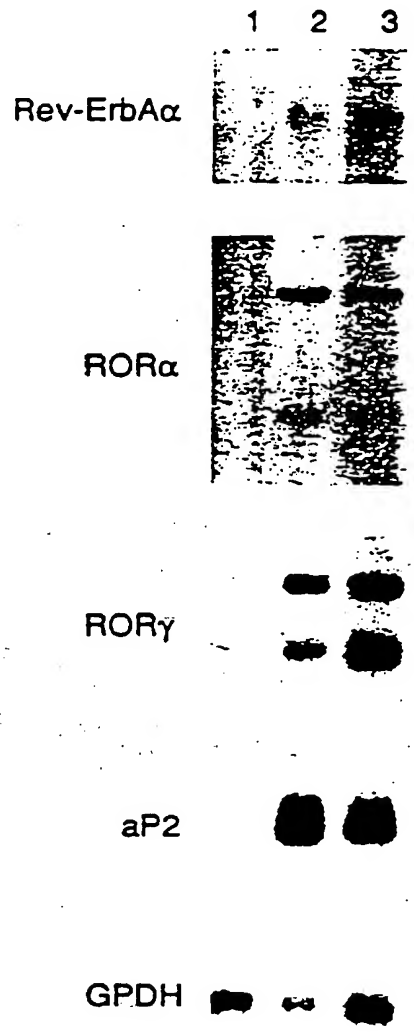

Fig. 6. Coexpression of RORα, RORγ, and Rev-ErbAα in D1 adipocytes. Cells were grown to confluence and then incubated in the absence (*Lane 1*) or presence of dexamethasone and insulin (*Lane 2*) or insulin and 100 μM clofibrate (*Lane 3*). In both cases, about 85% of the cells contained fat droplets. After 5 days, cells were collected and total RNA isolated. RNA was examined by Northern blot analysis using ^{32}P-labeled probes for GPDH, aP2, RORα, RORγ, and Rev-ErbAα.

to the transduced PPARγ gene, the endogenous PPARγ gene (Fig. 5). These cultures contained fat droplets (20–25% of the cells; data not shown) and did express aP2 mRNA, as reported previously (12). The induction of adipogenesis in these cells was accompanied by an increase in the levels of RORα and RORγ mRNA (Fig. 5). These results demonstrate that activation of PPARγ causes, either directly or indirectly, an induction of RORα and RORγ mRNA.

Cross-Talk between RORγ and Rev-ErbAα. Recently, the expression of another nuclear orphan receptor, Rev-ErbAα, was reported to be up-regulated during adipocyte differentiation of 3T3-L1 cells (21). As reported for 3T3-L1 cells, Rev-ErbAα mRNA is also induced during adipocyte differentiation of D1 cells (Fig. 6). In contrast to D1 cells maintained at confluence in the absence of differentiation inducers, confluent cultures treated with insulin plus dexamethasone or insulin plus clofibrate did express Rev-ErbAα mRNA, in addition to RORα and RORγ mRNA.

Because RORγ and Rev-ErbAα have been reported to bind to similar REs (33, 34), we investigated the possibility of an interaction between these two receptor signaling pathways by analyzing their binding and transactivation properties. Recently, we characterized the consensus RE optimal for binding RORγ, referred to as RORγ-RE, and showed that

it consists of TAAGTAGGTCAT (33). In Fig. 7, we compared the ability of RORα, RORγ, and Rev-ErbAα to bind to and transactivate through RORγ-RE. RORα, RORγ, and Rev-ErbAα were all able to bind to the RORγ-RE, as demonstrated by EMSA analysis (Fig. 7*A*). However, in contrast to RORα and RORγ, Rev-ErbAα was unable to induce transcription of the CAT reporter via RORγ-RE (Fig. 7*B*), in agreement with previous reports (33–35). These results imply that RORs and Rev-ErbAα would be able to compete with each other for binding to the same RE, and because Rev-ErbAα does not exhibit transactivation function, it would inhibit RORγ-mediated transactivation. The latter was verified by the experiment shown in Fig. 8*A*, which demonstrates that the RORγ-dependent transcription was repressed by increasing levels of Rev-ErbAα expression. These results indicate that, under the conditions used, the antagonism between Rev-ErbAα and RORγ is at least in part due to competition for the binding to RORγ-RE. This conclusion is in agreement with recent studies describing the dominant-negative action of Rev-Erb (34–38).

The interaction of nuclear receptors with the basal transcription machinery is dependent on its association with intermediary proteins with different functions, including repressor, coactivator, or integrator functions (39–43). These intermediary factors can interact with and compete for different nuclear receptors. Therefore, competition between Rev-ErbAα and RORγ for binding to common intermediary factors may be an additional mechanism involved in the suppression of the RORγ-induced transactivation by Rev-ErbAα. This hypothesis is supported by the experiment described in Fig. 8*B*, which shows that, in D1 cells, the induction of GAL4-dependent transcriptional activation of CAT, mediated by a GAL4(DBD)-RORγ fusion protein, can be suppressed by increasing levels of Rev-ErbAα expression. These results suggest that Rev-ErbAα can antagonize RORγ-mediated transactivation through a mechanism other than competition for RORγ-RE binding (Figs. 7 and 8*A*). The antagonistic effect of Rev-ErbAα on RORγ-induced transactivation may, in part, be explained by the squelching of transcription intermediary factors that are required for RORγ-mediated transactivation. Our results indicate that cross-talk between these orphan receptors may occur at more than one level.

We next analyzed the RORγ-RE-dependent transactivation of the CAT reporter gene by endogenous nuclear receptors at different stages of differentiation of D1 cells. As shown in Fig. 9, the RORγ-RE-dependent transactivation increased dramatically after 2 days of treatment with differentiation inducers. This time interval coincides with the increase in RORα and RORγ expression observed in Fig. 3. These results indicate that transactivation through RORγ-RE is regulated in a differentiation-dependent manner. This enhanced transactivation might at least in part be attributed to increased RORα and RORγ expression.

Discussion

Many nuclear receptors play an important role in the control of gene expression during cellular differentiation (9, 10). To determine the functions of the nuclear orphan receptor

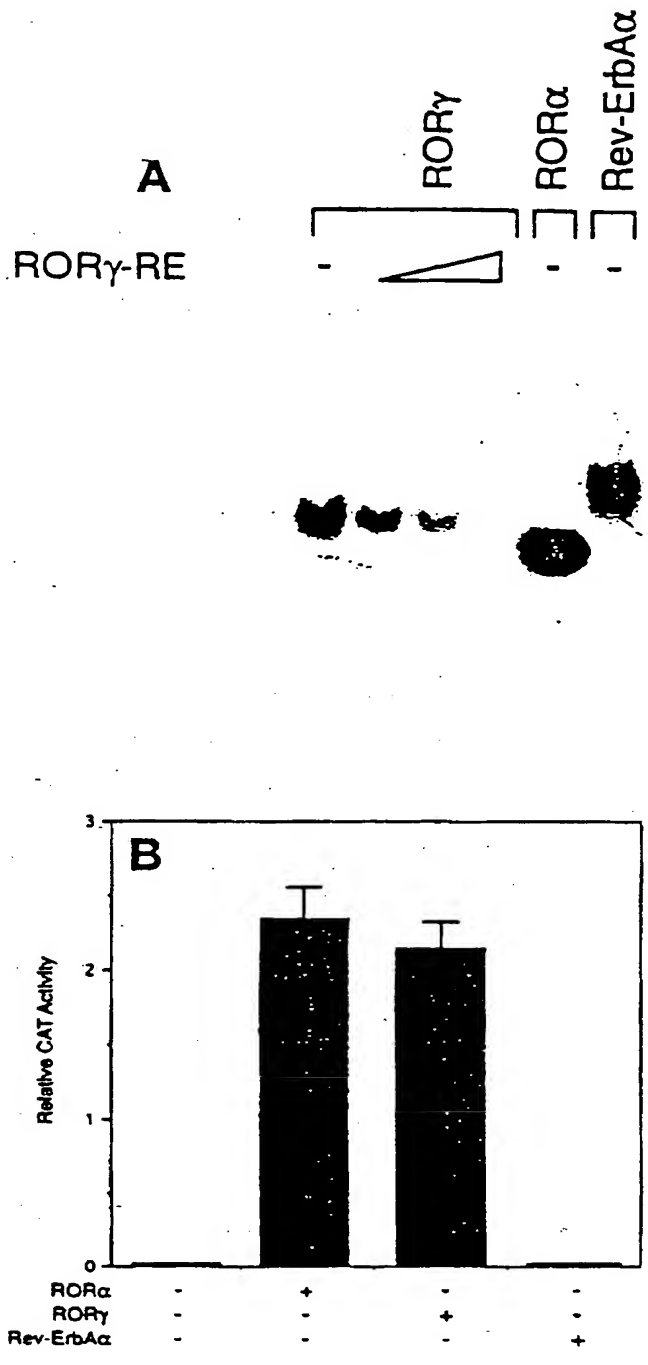

Fig. 7. Comparison of RORγ-RE-dependent transactivation and RORγ-RE binding by RORα, RORγ, and Rev-ErbAα in preadipocyte D1 cells. *A*, binding of *in vitro* translated RORα, RORγ, and Rev-ErbAα to ^{32}P-labeled RORγ-RE was determined by EMSA. The samples in the *second through fourth lanes* were incubated in the presence of 5-, 25-, and 100-fold excess of unlabeled RORγ-RE, respectively. *B*, cells were transiently transfected with β-actin-LUC, the $(RORγ\text{-}RE)_4$-tk-CAT reporter plasmid and an expression vector encoding RORα, RORγ, or Rev-ErbAα using Lipofectamine. After 48 h, cells were collected and assayed for CAT and luciferase activity, as described in "Materials and Methods." *Columns*, relative CAT activities. *Bars*, SD.

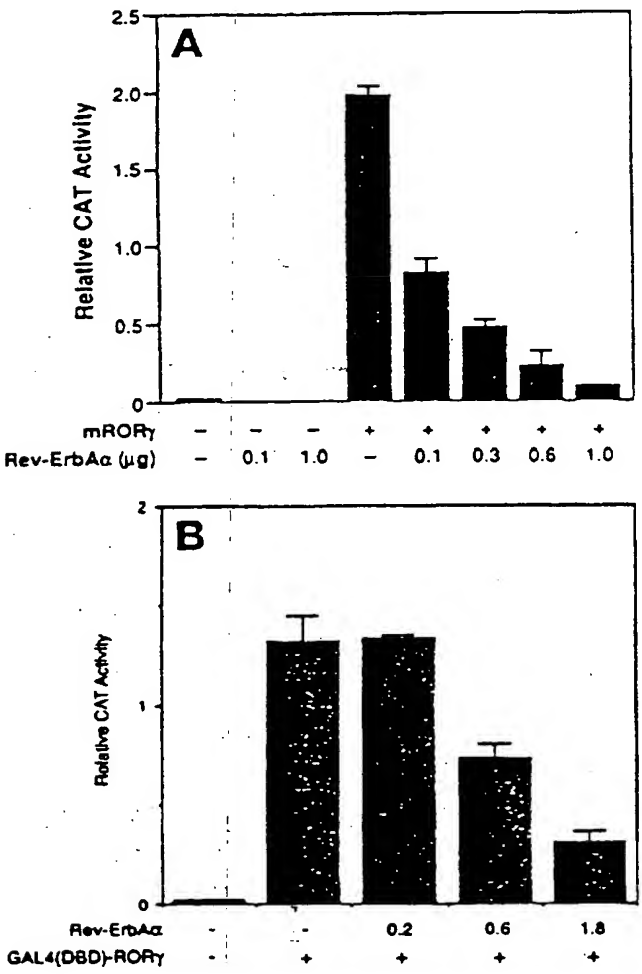

Fig. 8. Cross-talk between the RORγ and Rev-ErbAα receptor signaling pathways in preadipocyte D1 cells. *A*, inhibition of RORγ-RE-dependent transactivation by Rev-ErbAα. Cells were plated in growth medium and were transfected the next day with β-actin-LUC, the $(ROR\gamma\text{-}RE)_4$-tk-CAT reporter plasmid, and an expression vector encoding RORγ using Lipofectamine, as described in "Materials and Methods." Cells in separate dishes were cotransfected with increasing amounts of the Rev-ErbAα expression plasmid. *B*, inhibition of GAL(DBD)-RORγ mediated transactivation by Rev-ErbAα. D1 cells were transfected with GAL(DBD)-RORγ, pG5CAT, and with β-actin-LUC, as described above. Cells in separate dishes were cotransfected with increasing amounts (μg) of Rev-ErbAα expression plasmid. Forty-eight h after transfection, cells were collected and assayed for CAT and luciferase activity. *Columns*, relative CAT activities. *Bars*, SD.

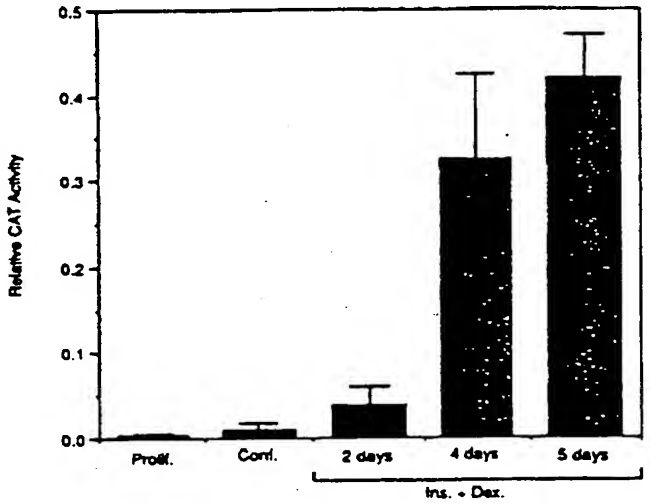

Fig. 9. RORγ-RE-dependent transactivation by endogenous nuclear receptors. D1 cells in the exponential growth phase (*Prolif.*), confluent cultures (*Conf.*), and confluent cultures at different stages of differentiation [treated with insulin and dexamethasone (*Ins. + Dex.*) for 2, 4, and 5 days] were transiently transfected with β-actin-LUC and the $(ROR\gamma\text{-}RE)_4$-tk-CAT reporter plasmids using Lipofectamine. Cells were collected and assayed for CAT and luciferase activity, as described in "Materials and Methods." *Columns*, relative CAT activities. In a separate control experiment, tk-CAT was measured at different time points during adipocyte differentiation and found to remain low and not change significantly (less than 2-fold) over the time course. *Bars*, SD.

RORγ, we examined its expression during cellular differentiation of several *in vitro* cell systems. Here, we describe the differential regulation of RORγ expression during adipocyte differentiation and provide evidence for cross-talk between RORγ and other nuclear receptor signaling pathways. RORγ mRNA is undetectable in undifferentiated D1 and 3T3-L1 preadipocytes and becomes expressed when these cells undergo differentiation into fat cells. RORγ is not expressed in Swiss and BALB/c3T3 cells, which do not undergo differentiation, grown either in the exponential growth phase or under conditions that allow adipogenesis in D1 and 3T3-L1 cells. RORα showed a very similar pattern of induction during adipocyte differentiation as RORγ; however, in contrast to RORγ, it was expressed at about the same level in both logarithmic and confluent cultures of Swiss 3T3 cells. The link between RORα and RORγ expression and the adipocyte phenotype was confirmed by the observed inhibition of RORγ mRNA induction by TNF-α and TGF-β, two known inhibitors of adipocyte differentiation (3–6). Moreover, we recently reported that the repression of adipocyte differentiation by retinoids is accompanied by inhibition of PPARγ, RORα, and RORγ expression (44). In addition, thiazolidinediones, PGD_2, or 15-deoxy-$\Delta^{12,14}$-PGJ_2, which have been reported to activate PPARγ and induce fat cell differentiation in the mesenchymal stem cells C3H10T1/2 (15–17), increase RORγ mRNA expression (Fig. 4). The expression and activation of PPARγ has been demonstrated to play a critical role in the commitment of cells to undergo adipocyte differentiation (12–14). This became evident in studies showing that ectopic expression of PPARγ in BALB/c3T3 cells enables them to undergo adipocyte differentiation after treatment with clofibrate (12). As shown in Fig. 5, activation of PPARγ in these cells results in an induction of RORα and RORγ mRNA. Whether RORα or RORγ are putative target genes for PPARγ and/or C/EBPα regulation awaits the characterization of the regulatory regions of these genes.

Analysis of the time course of the induction of RORα and RORγ mRNA indicates that the expression of these orphan receptors is increased at a very similar time as aP2, a late marker for adipocyte differentiation. These results show that the induction of RORα and RORγ is not an early event and

suggest that these receptors may play a role in the modulation of gene expression at a late stage of adipocyte differentiation rather than in the commitment or initiation of adipocyte differentiation.

The members of the ROR subfamily have been reported to bind as monomers to REs that consist of a single core motif preceded by a 6-bp A+T-rich region (30, 32, 33). Recently, we characterized the consensus sequence of the RE (RORγ-RE) for the RORγ receptor (33) and showed that it consists of AATCAAGGTCA. RORγ is able to induce RORγ-RE-dependent transactivation of the CAT reporter gene in preadipocyte D1 cells very effectively. RORα, which is also induced during adipocyte differentiation, can also bind RORγ-RE and increase RORγ-RE-dependent transactivation in D1 cells. Although RORα and RORγ can bind to similar elements, these receptors appear to possess different affinities for slightly different RORs, resulting in the regulation of different target genes. It is likely that the transcriptional activity of RORs is controlled by their interaction with a ligand and/or posttranscriptional modifications through second messenger-activated signaling pathways. Identification of the signal or ligand that modulates the activity of RORγ will provide an important tool in elucidating the mechanism by which RORγ controls gene expression.

Cross-talk between different nuclear receptor signaling pathways has been demonstrated for various members of the nuclear receptor superfamily (12, 37, 45). Here, we provide evidence for an interaction between the RORγ and Rev-Erb signaling pathways. We show that, in addition to RORα and RORγ, differentiating D1 cells start to express the nuclear orphan receptor Rev-ErbAα, in agreement with previous observations in 3T3-L1 cells (21). Whereas all three receptors are able to bind to the RORγ-RE, as shown by EMSA, only RORα and RORγ induce RORγ-RE-dependent transcription of a CAT reporter gene in preadipocyte D1 cells. Rev-ErbAα appears to be transcriptionally inactive (Fig. 8*B*) and is able to repress the transactivation mediated by RORα and RORγ in preadipocyte D1 cells. The antagonism between these receptor signaling pathways is likely due, at least in part, to competition for binding to the RORγ-RE. This conclusion is in agreement with the reported inhibition of RORα-mediated transcriptional activation by Rev-Erb in CV-1 cells (37) and its dominant-negative effect in C2C12 cells (35, 38). The transcriptional repression by Rev-ErbAα has been shown to involve the signature motif and helix 5 in its ligand-binding domain (46). Our results also suggest that Rev-ErbAα could inhibit RORγ-mediated transactivation through an alternative mechanism that involves competition for binding to common transcription intermediary factors (squelching).

The RE (RORγ-RE) used in this study was selected for optimal binding to RORγ (33). However, the REs in target genes are variable and likely exhibit different affinities for RORα and RORγ and Rev-ErbAα. Differences in DNA-binding activity between these receptors might determine whether Rev-ErbAα is able to antagonize the transactivation of a particular gene by RORs or not. Such differences may also be important in determining which genes are regulated by RORα or RORγ. The observed correlation between the increase in RORγ-RE-dependent transactivation and RORα and RORγ expression during differentiation of D1 cells (Figs. 3 and 9) appears to support the concept that this type of transactivation plays a role in the regulation of specific genes at later stages of adipocyte differentiation. In addition, it suggests that the antagonism between ROR and Rev-ErbAα may only be involved in the regulation of certain specific genes, of which the RE has a higher affinity for Rev-ErbAα than do RORs. The importance of these opposing receptor activities may become clear when target genes for these receptors are identified.

Materials and Methods

Materials. Human TNF-α and human TGF-β1 were purchased from R&D Systems (Minneapolis, MN). The antidiabetic drugs pioglitazone and BRL49653 were obtained from Dr. J. Lehmann (Glaxo-Wellcome, Research Triangle Park, NC). PGD_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 were purchased from Cayman Chemical (Ann Arbor, MI).

Cell Culture. Mouse fibroblast D1 cells were described previously (47). Balb/3T3, Swiss 3T3, NIH3T3, C3H10T1/2, and 3T3-L1 cells were obtained from American Type Culture Collection (Rockville, MD). The mouse myoblast cell line 3T3-C2C12 was obtained from American Type Culture Collection. All cells were grown in RPMI 1640 or DMEM containing 10% fetal bovine or calf serum (Atlanta Biologicals, Atlanta, GA) in the presence of penicillin and streptomycin. To induce adipocyte differentiation, cultures were grown to confluence and then treated with 1 μM dexamethasone and 10 μg/ml insulin, unless stated otherwise. In certain instances, cells were treated with dexamethasone, insulin, and 0.5 mM IBMX. The number of adipocytes were determined after Oil Red O staining.

Plasmids. The expression vectors pCMXRORα1 and Rev-ErbAα were obtained from Drs. V. Giguère (McGill University, Montreal, Quebec, Canada) and M. Lazar (University of Pennsylvania, Philadelphia, PA), respectively. The plasmid mRORγ-pZeoSV was constructed by inserting a *SpeI-XhoI* fragment of mRORγ-BSK (33) into the expression vector pZeo-SV (Invitrogen, San Diego, CA). The reporter plasmid $(RORγ\text{-}RE)_4$-tk-CAT was constructed by inserting a chemically synthesized oligonucleotide containing four repeats of the consensus RORγ-RE, $(GGTAAGTAGGTCAT)_4$, into the pBLCAT5 vector (48). The expression vector GAL4(DBD)-RORγ encoding the fusion protein GAL4(DBD)-RORγ (amino acids 40–516) was constructed by cloning the *BamHI-HindIII*, 1.8-kb fragment of mRORγ, including the hormone-binding domain, into the pM expression vector (Clontech, Palo Alto, CA) containing the GAL4(DBD). GAL4(DBD), containing amino acids 1–147 of the yeast transcriptional factor GAL4 (including the DBD), was linked to the NH_2 terminus of the truncated mRORγ.

Northern Blot Analysis. Total RNA was isolated from cultured cells using Tri Reagent (Sigma Chemical Co., St. Louis, MO), according to the manufacturer's protocol. Total RNA (30 μg) was electrophoresed through a formaldehyde agarose gel as described (49, 50), blotted to a Nytran membrane (Schleicher & Schuell, Keene, NH), and UV cross-linked. Hybridizations were performed for 1–2 h at 68°C using QuikHyb reagent (Stratagene, La Jolla, CA), and blots were washed twice with 2× SSC-0.05% SDS at room temperature for 15 min. The final wash was with 0.5× SSC-0.1% SDS at 65°C for 30 min. Autoradiography was carried out with Kodak X-Omat AR film at −70°C using double intensifying screens. The mRORγ was excised from pBluescript S/K− clone 5 containing the full coding region of mouse RORγ (33). Plasmids containing inserts encoding aP2, PPARγ, LPL, and C/EBPα were obtained from Drs. B. M. Spiegelman (Dana-Farber Institute, Boston, MA) and R. Brent (NIH, Bethesda, MD) and K. G. Xanthopoulos (NIH, Bethesda, MD), respectively. A 1.26-kb fragment of the chicken GPDH gene (51) was used as a control probe. Expression plasmids containing Rev-ErbAα or RORα1 were kindly provided by Drs. M. A. Lazar and V. Giguère, respectively.

RT-PCR. Because detection of RORα mRNA by Northern blot analysis required long exposure times (2 weeks), an RT-PCR assay was developed and used in later studies. RT-PCR was carried out using 0.1 μg of total RNA and a GeneAmp® RNA PCR kit (Perkin-Elmer Corp., Foster City, CA) and RORα-specific primers 5′-GCTTCTACCTGGACATC (upstream) and

5'-ACACAGCTGCCACATCACCT (downstream) and the GPDH-specific primers 5'-TGAAGGTCGGTGTGAACGG-ATTTGGC and 5'-CATGTAG-GCCATGAGGTCCACCAC, obtained from Clontech. PCR was performed at 2.0 mM $MgCl_2$ for 30 cycles.

Transient Transfection. Preadipocyte D1 cells were trypsinized and plated in six-well dishes in complete growth medium. The next day, cells were cotransfected with 1.0 μg of $(ROR\gamma\text{-}RE)_4$-tk-CAT and 0.5 μg of mRORγ-pZeoSV, pCMXRORα1, or Rev-ErbAα, unless stated otherwise, using Lipofectamine (Life Technologies, Inc.) in medium without antibiotics and serum. β-Actin-LUC plasmid DNA (0.5 μg) was used as an internal control to monitor transfection efficiency. After 5 h, medium was removed, and cells were incubated in complete growth medium for another 48 h. Cells were harvested and assayed for luciferase and CAT activity. Luciferase activity was determined using the luciferase assay kit from Promega (Madison, WI). CAT activity was determined using a CAT-ELISA kit from Boehringer-Mannheim (Indianapolis, IN). All experiments were done at least twice and performed in triplicate. In a second series of experiments, GAL4(DBD)-RORγ plasmid DNA was transfected in fibroblast D1 cells, as described above, with pG5CAT (Clontech), which contains five GAL4 binding sites in front of the E1B minimal promoter and CAT reporter gene. In separate dishes, cells were cotransfected with the Rev-ErbAα expression vector.

EMSA. The SP6 RNA coupled reticulocyte lysate system (Promega) was used to synthesize mRORγ protein from mRORγ-pZeoSV. The T7 RNA coupled reticulocyte lysate system was used to synthesize RORα and Rev-ErbAα protein from pCMXRORα1 and Rev-ErbAα. The RORγ-RE was end-labeled with ^{32}P-γ-ATP by T4 polynucleotide kinase (Promega). Approximately 0.2–0.5 ng (50,000 cpm) of the probe was used in a binding reaction with 2 μl of programmed reticulocyte lysate in a buffer containing 20 mM HEPES (pH 7.9) 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, and 10% glycerol. To prevent nonspecific binding, 1 μg of poly(dI-dC), 1 μg of salmon sperm DNA and 0.5 μg of single-strand, nonspecific oligonucleotide were included in the reaction buffer. The programmed lysates were first incubated with reaction buffer for 10 min at room temperature and then for 30 min in the presence of the radiolabeled probe, with or without competitor. The receptor-nucleotide complexes were separated on a 5% nondenaturing polyacrylamide gel containing 0.5× Tris-borate EDTA.

Stable Transfection of PPARγ. The expression plasmids pBABE and pBABE-PPARγ were kindly provided by Dr. B. Spiegelman. BOSC23 cells (Dr. D. Baltimore, Rockefeller University, New York, NY) were transfected with pBABE-PPARγ or pBABE using the calcium phosphate coprecipitation procedure. Viral supernatants were harvested 48 h after transfection, as described previously (12). BALB/c3T3 cells were infected with retroviruses and then selected in medium containing puromycin (5–8 μg/ml). Selected cells, BALB/c3T3-pBABE, and BALB/c3T3-PPARγ were grown to confluence and then treated with insulin and dexamethasone in the presence or absence of 100 μM clofibrate (Sigma).

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A functional Rev-erbα responsive element located in the human Rev-erbα promoter mediates a repressing activity

(nuclear receptors/gene expression)

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ABSTRACT **Rev-erbα belongs to the nuclear receptor superfamily, which contains receptors for steroids, thyroid hormones, retinoic acid, and vitamin D, as well as "orphan" receptors. No ligand has been found for Rev-erbα to date, making it one of these orphan receptors. Similar to some other orphan receptors, Rev-erbα has been shown to bind DNA as a monomer on a specific sequence called a Rev-erbα responsive element (RevRE), but its transcriptional activity remains unclear. In this paper, we characterize a functional RevRE located in the human Rev-erbα promoter itself. We also present evidence that (*i*) Rev-erbα mediates transcriptional repression of its own promoter *in vitro*, (*ii*) this repressing effect strictly depends on the binding of Rev-erbα to its responsive element and is transferable to a heterologous promoter; and (*iii*) Rev-erbα binds to this responsive sequence as a homodimer.**

Nuclear receptors are transcription factors that provide a direct link between an extra- or intracellular signaling molecule and the transcriptional response (1). These nuclear receptors have a structural organization based on modular domains: from the amino to the carboxyl terminus, these are the variable A/B domain important for transactivation; the highly conserved C domain involved in DNA binding and dimerization; the D domain, which is a flexible hinge; and the large and moderately conserved E domain, which carries out several functions such as ligand binding, dimerization, and ligand-dependent transcriptional regulation. In addition to receptors for known ligands, this superfamily also contains receptors for which no specific ligand has yet been found (1, 2). These "orphan" receptors could either be receptors for ligands that have yet to be discovered or receptors that actually have no ligand and act as constitutive transcription factors, which would be controlled, like NGFIB, at the expression level (3). Whereas most nuclear receptors bind DNA as homo- or heterodimers to tandem repeats of the core sequence AGGTCA (1, 4–6), an increasing number of orphan receptors (NGFIB, FTZ-F1, RZR) have been shown to bind preferentially to their response element as monomers (7–11).

The Rev-erb genes encode two highly related orphan receptors named Rev-erbα and Rev-erbβ. To date, little is known about their function: Rev-erbα is expressed in numerous adult tissues and is induced during adipocyte differentiation (12). In contrast, Rev-erbβ is mainly expressed during the development of the nervous system, with a striking distribution in the floor plate of the neural tube (13). It has recently been shown that both Rev-erbα and Rev-erbβ bind to the Rev-erbα responsive element RevRE: $\underset{T}{\overset{A}{-}}A\underset{T}{\overset{A}{-}}NT\underset{G}{\overset{A}{-}}$GGTCA) as a monomer (14). However, contrary to other orphan receptors for which the transcriptional activity is well established (NGFIB, FTZ-F1, or RZR), this function remains controversial for Rev-erbα (14–18).

To gain insight into this important aspect of Rev-erbα function, we studied its transcriptional activity on the natural RevRE found in the human Rev-erbα promoter gene. In this paper, we show that through this RevRE, either placed in its natural promoter context or when transferred upstream of the simian virus 40 (SV40) promoter, both Rev-erbα and Rev-erbβ exhibit transcriptional repressing activity. Furthermore, we show that Rev-erbα is able to bind to this responsive element as a homodimer.

MATERIALS AND METHODS

Primer Extension Analysis. Primer extension analysis was performed following ref. 19. Briefly, 5 μg of total HepG2 RNA was denatured at 80°C for 3 min and annealed at 65°C to a 5′-end-labeled primer. Reverse transcription was carried out at 37°C for 45 min with 500 units of Moloney murine leukemia virus reverse transcriptase (BRL). Extended fragments were resolved on a 6% denaturing gel.

Constructs. The 2-kb *Eco*RI–*Bam*HI genomic fragment was prepared from the λE2A3 genomic phage by partial digestion and subcloned and sequenced into the pBluescript II SK (+) vector (Stratagene), giving rise to the 2-kb pBSK construct.

pRev-erbαWT was constructed as follows: a 1.46-kb *Eco*RV–*Sst* I fragment of the 2-kb pBSK construct was cloned between the *Sma* I and *Sst* I sites of the pGL2-Basic vector (Promega), giving rise to 1.46-kb pGL2. The 3′ portion of the promoter, extending from nt 1430 to nt 1733 was amplified by PCR and cloned into the pGEM-T vector (Promega). The resulting construct was digested with *Sst* I and *Sal* I and was inserted between the *Sst* I and *Xho* I sites of the 1.46-kb pGL2 construct, giving rise to pRev-erbαWT. pRev-erbαΔ and pRev-erbαCCC were constructed following the same strategy except that the desired Rp mutation was introduced by internal mutated primers during the PCR.

The pRev-erbαδ1 and pRev-erbαδ2 vectors were constructed by PCR: promoter fragments, extending from nt 975 to nt 1733 and from nt 1038 to nt 1733, respectively, were amplified by PCR and cloned into the pCRII vector (Invitrogen). *Eco*RV–*Bam* HI fragments were prepared from these pCRII vectors and subcloned into the pGL2-Basic vector digested with *Sma* I and *Bgl* II. pRev-erbαΔδ1 was constructed by replacing the *Sst* I–*Kpn* I fragment of pRev-erbαδ1 by the *Sst* I–*Kpn* I fragment prepared from pRev-erbαΔ.

pRev-erbαWTinv was constructed by subcloning the 1.64-kb *Xho* I–*Ssp* I fragment of the 2-kb pBSK construct between the *Sma* I and *Xho* I sites of the pGL2-Basic vector. pRev-erbαδAsp and pRev-erbαδSsp were derived from pRev-

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Abbreviations: RevRE, Rev-erbα responsive element; SV40, simian virus 40.

The sequence reported in this paper has been deposited in the GenBank data base (accession no. X95536).

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erbαδ1 digested, respectively, with *Xho* I and *Asp*718 or with *Xho* I and *Ssp* I sites that were blunt-ended before ligation.

pWT-SV40 and pΔ-SV40 were obtained by subcloning *Sst* I–*Asp*718 fragments prepared, respectively, from pRev-erbαWT and pRev-erbαΔ between the *Sst* I and *Xho* I sites of the pGL2-Promoter vector (the *Asp*718 and *Xho* I sites were blunt-ended).

pRevDR2, pRevDR2M5′, and pRevDR2M3′ were constructed as follows: the following oligonucleotides carrying *Bam*HI and *Bgl* II restriction sites were concatemerized (mutated nucleotides are underlined) and ligated into the *Bam*HI site of pBluescript II SK (+).

5′-GATCCGGAAAAGTGTGTCACTGGGGCACGA-3′ (RevDR2 sense+)
5′-GATCTCGTGCCCCAGTGACACACTTTTCCG-3′ (RevDR2 sense−)
5′-GATCCGGAAAAGTCCTAGCCTGGGGCACGA-3′ (RevDR2M5′ sense+)
5′-GATCTCGTGCCCCAGGCTAGGACTTTTCCG-3′ (RevDR2M5′ sense−)
5′-GATCCGGAAAAGTGTGTCACTCCTAGCCGA-3′ (RevDR2M3′ sense+)
5′-GATCTCGGCTAGGAGTGACACACTTTTCCG-3′ (RevDR2M3′ sense−)

The number and orientation of cloned monomers were determined by sequencing. Dimers were subcloned between the *Kpn* I–*Sst* I sites of the pGL2-Promoter vector.

pREVERBα was constructed by inserting the 2.1-kb *Eco*RI–*Bam*HI fragment from pSP65 between the *Eco*RI and *Bam*HI sites of pSG_5 (20). pREVERBα-(1–236) was constructed by replacing the *Xho* I–*Bgl* II fragment of pREVERBα with an *Xho* I–*Bgl* II PCR fragment coding for a domain from Ser-200 to Pro-236 that carried an in-frame stop codon.

pSVREVFull and pSVREV236 are pSG_5-based vectors, encoding proteins extending from Thr-2 to Glu-614 and from Thr-2 to Pro-236, respectively, fused to a peptide composed of the hemagglutinin epitope and the SV40 nuclear localization signal.

All PCR-derived constructs were sequenced.

Cell Transfection and Reporter Assays. One day before transfection, one confluent 100-mm dish of HepG2 cells was split onto two six-well plates. Plasmid DNA was transfected using Lipofectamine (BRL). The transfection medium was replaced by DMEM containing 10% fetal calf serum (BRL) 6 h after addition of the liposome–DNA complex to the cells. Cells were lysed 48 h or 72 h after transfection (as indicated), and an aliquot was assayed for luciferase activity. Cotransfected pRSVCAT allowed transfection normalization. In all cases, chloramphenicol acetyltransferase (CAT) activity did not vary by more than 15% from point to point within a single experiment. All transfection experiments were performed at least three times and gave similar results. Results are presented as the mean ± SD.

Electrophoretic Mobility Shift Assay. The 73-bp WT and Δ probes were prepared from pRev-erbαWT and pRev-erbαΔ digested with *Sst* I and *Asp*718. RevDR2, RevDR2M5′, and RevDR2M3′ oligonucleotides are described above. The probes were labeled using the Klenow fragment of DNA polymerase or polynucleotide kinase and purified on a polyacrylamide gel. Proteins were expressed using the TNT reticulocyte lysate kit (Promega). The protein–DNA interactions were carried out for 30 min at 4°C with 0.5 ng of probe (≈20,000 cpm) in the following buffer: 0.5% glycerol, 60 mM KCl, 1 μg of bovine serum albumin, 30 mM Hepes (pH 7.5), 1 mM dithiothreitol, 0.1% Triton X-100, and 60 ng of poly(dI-dC). Competition assays were carried out with the following oligonucleotides: WT, 5′-CGCGTGAAAAGTGTGTCAGATCT-3′ (sense +) and 5′-GATCTGACACACTTTTCA-3′ (sense −); Δ, 5′-CGCGTGAAAAGTCCTAGCAGATCT-3′ (sense +) and 5′-GATCTGCTAGGACTTTTCA 3′ (sense −). Competition oligonucleotides were added simultaneously with the labeled probes at the indicated molar excess. Complexes were run on a 4% acrylamide gel containing 0.1% Triton X-100 for 3 h at 200 V at 4°C.

RESULTS

Characterization of the Human Rev-erbα Promoter. To isolate the Rev-erbα promoter, we established a restriction map of the λE2A3 phage that contains the 5′ portion of the published human Rev-erbα cDNA (20–22). The 5′ region of this phage contains a 740-bp *Eco*RI fragment linked 5′ to a

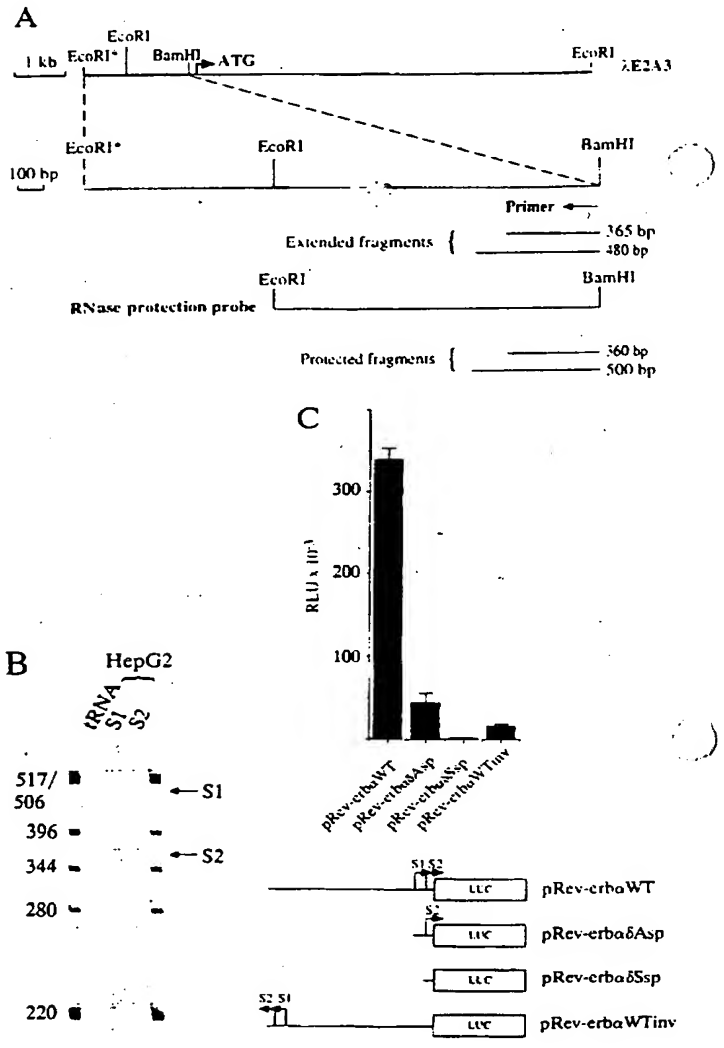

FIG. 1. Characterization of the human Rev-erbα promoter. (*A*) Localization of the 2-kb *Eco*RI–*Bam*HI fragment within the λE2A3 genomic phage. The primer used for primer extension and the RNase protection probe are depicted. The resulting extended fragments and protected fragments are shown. (*B*) Primer extension experiment. Two samples of HepG2 total RNA, as well as a yeast tRNA control, were reverse-transcribed from the primer that hybridizes to the *Bam*HI site (see *A*). Two extended fragments (S1 and S2) of 480 bp and 365 bp were obtained. (*C*) Promoter activity of the wild type (full length) and of start site deletion mutants of human Rev-erbα promoter. HepG2 cells were transfected with 5 μg of the indicated luciferase reporter constructs together with 0.25 μg of pRSVCAT as an internal standard. The resulting luciferase activity was measured 72 h after transfection. RLU, relative light units; LUC, luciferase reporter gene.

1.26-kb *Eco*RI–*Bam*HI fragment encompassing the 5′ end of the cDNA. The initiation codon is located 123 bp downstream from this *Bam*HI site (Fig. 1*A*). The 5′ boundary of Rev-erbα exon 1 was mapped by primer extension experiments using a primer containing this *Bam*HI site (Fig. 1*A*). Two specific bands were reproducibly obtained with HepG2 total RNA but not with the control yeast tRNA (Fig. 1*B*). These two major start sites, called S1 and S2, are located 480 bp and 365 bp, respectively, 5′ to the primer. The location of these start sites was confirmed by RNase protection (Fig. 1*A* and data not shown).

To further confirm the location of these transcription start sites, we placed the 5′ part of this genomic sequence, extending from the 5′-most *Eco*RI site to nt 1733 (see Fig. 2), in front of the luciferase reporter gene to yield the pRev-erbαWT construct. When transfected into HepG2 hepatocarcinoma cells, this construct exhibited a strong luciferase activity as compared to a control construct containing this region cloned in the inverted orientation (pRev-erbαWTinv; Fig. 1*C*). In addition, a 5′ deletion construct, pRev-erbαδAsp, in which the transcription start site S1 is deleted, retained a weak luciferase activity, while the pRev-erbαδSsp construct, in which both S1 and S2 are deleted, was devoid of luciferase activity (Fig. 1*C*). The promoter was also active, although to a lesser degree, in HeLa cells (data not shown). These results demonstrate that this 2-kb genomic region functions as a promoter. This sequence harbors two putative TATA box sequences that could

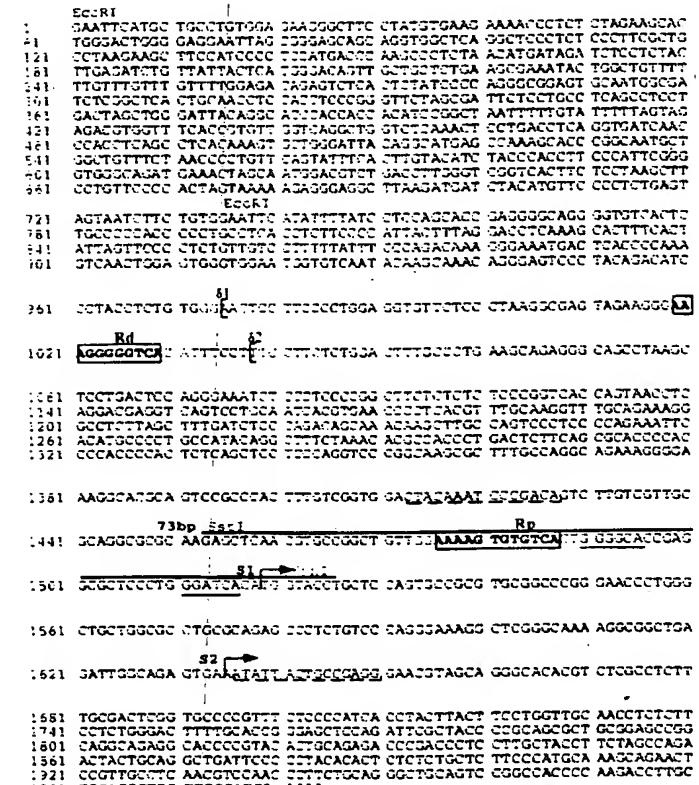

FIG. 2. Sequence of the human Rev-erbα promoter. The S1 and S2 start sites are indicated by bent arrows. The putative transcriptional initiation motifs are underlined with a dashed line. pRev-erbαWTδ1 and pRev-erbαWTδ2 5′ borders are indicated by brackets. The star indicates the 3′ border of all promoter constructs. Rd and Rp sites are indicated as shaded boxes. A thick line is drawn above the sequence of the 73-bp probe. The two degenerate AGGTCA sequences located downstream of the Rp site are underlined. The human Rev-erbα promoter sequence has been deposited in the GenBank data base (accession number X95536).

be relevant to the mapped starts sites (shown as dashed line in Fig. 2). It also contains consensus binding sites for numerous transcription factors among which we noticed the presence of putative binding sites for nuclear receptors. Interestingly, two of these AGGTCA-like motifs, called Rp and Rd, contain at their 5′ ends an A/T-rich sequence (see Fig. 2). According to recently reported *in vitro* experiments, such sites could represent RevREs (14).

Rev-erbα Represses the Human Rev-erbα Promoter. To investigate the role of these putative binding sites, the pRev-erbαWT promoter construct and the pRev-ERBα expression vector were cotransfected into HepG2 cells. As shown in Fig. 3*A*, the Rev-erbα promoter activity was markedly repressed by the Rev-erbα protein. To identify which of the two putative binding sites mediated the Rev-erbα effect, we tested two 5′-deletion mutants for their response to Rev-erbα cotransfection: pRev-erbαδ1, which contained both the distal (Rd) and the proximal (Rp) binding sites but lacked the 5′ promoter sequence, and pRev-erbαδ2, which contained only the proximal binding site (Rp). As both of these deletion constructs still responded to the same extent as the wild-type promoter to pREV-ERBα cotransfection, we concluded that this repression was only mediated by the proximal binding site (Fig. 3*A*).

To investigate the role of the proximal Rev-erbα binding site in this regulation, we constructed two mutants of this site: one mutant (pRev-erbαΔ) is altered in the entire AGGTCA core sequence, whereas the other (pRev-erbαCCC) is only altered in the 5′ A/T-rich region that has been previously shown to be important for Rev-erbα binding (13–15). As exemplified in Fig. 3*B*, either alteration of this proximal binding site drastically reduced Rev-erbα-mediated repression. Similar effects were also observed upon cotransfection of Rev-erbβ, a close relative to Rev-erbα that exhibits the same binding specificity (Fig. 3*B*; refs. 13–15). As shown in Fig. 3*B*, a shorter mutant construct deleted of the 5′-most promoter sequence (pRev-erbαΔδ1) was not repressed by Rev-erbα, establishing that the remaining repression observed on pRev-erbαΔ was mediated through a cryptic Rev-erbα binding site located 5′ to Rd. Taken together, these results demonstrate that the proximal Rev-erbα binding site of the human Rev-erbα promoter acts as a negative responsive element.

The Repressing Effect of Rev-erbα Is Transferable to a Heterologous Promoter. To test whether the Rev-erbα repression was transferable to a heterologous promoter, a 73-bp fragment of the Rev-erbα promoter encompassing the Rp site either in its wild-type (WT) or mutated (Δ) version was inserted upstream of the SV40 promoter (Fig. 2). As shown in Fig. 4, the pWT-SV40 construct was significantly repressed upon cotransfection of the Rev-erbα expression vector. Conversely, neither the control pSV40 construct nor the pΔ-SV40 construct carrying a mutated Rp site was repressed by Rev-erbα. These results, which were also obtained in rabbit kidney RK13 cells (data not shown), strongly suggest that Rev-erbα is able to repress the activity of a heterologous promoter through the Rp site.

Rev-erbα Binds the Rp Site as a Homodimer. We next investigated the binding characteristics of Rev-erbα to this promoter region. *In vitro*-synthesized Rev-erbα protein (and Rev-erbβ; data not shown) was incubated with the labeled *Sst* I–*Kpn* I promoter fragment that encompasses the Rp site, either in its wild-type (WT73bp) or mutated (Δ73bp) version. As shown in Fig. 5*A*, Rev-erbα strongly bound to the WT73bp probe, whereas no binding could be observed when the Rp site was mutated (Fig. 5*A*). These data were confirmed by competition experiments where the resulting complex was efficiently blocked by competition with an unlabeled consensus RevRE oligonucleotide but not by its mutant version (Fig. 5*A*). Taken together, these binding experiment results corroborate the transactivation data (Figs. 3 and 4).

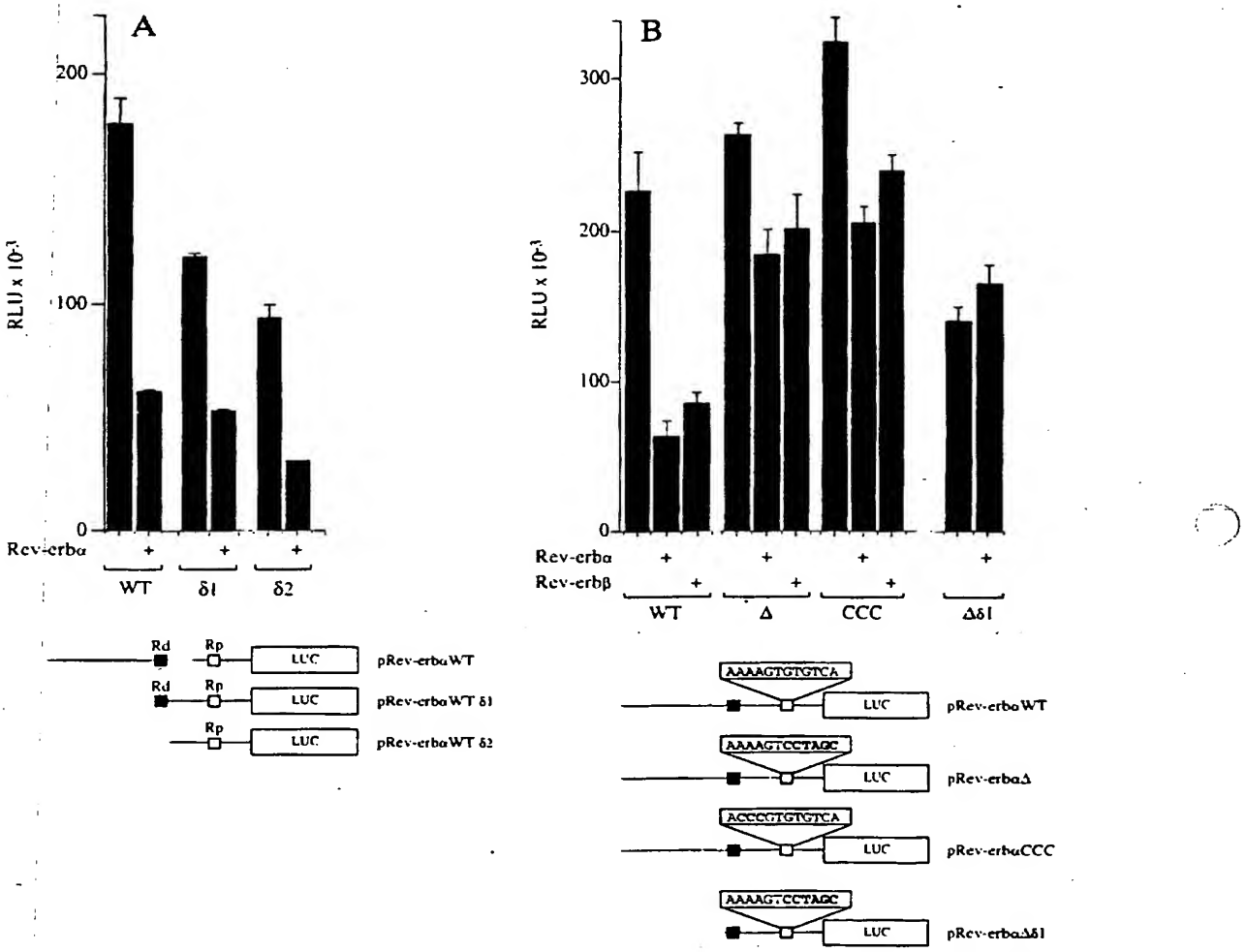

FIG. 3. (*A*) Determination of the functional Rev-erbα binding site. HepG2 cells were cotransfected with 1 μg of the pSG_5 or pREV-ERBα expression vectors and 4 μg of each of the human Rev-erbα promoter constructs shown schematically below the graph. The boxes represent Rd and Rp binding sites as labeled. (*B*) Rev-erbα repression of the promoter constructs mutated in the Rp motif. HepG2 cells were cotransfected with 1 μg of either pSG_5, pREV-ERBα, or pREV-ERBβ together with 4 μg of the promoter constructs depicted below the graph. The Rd and Rp sites are shown as solid and shaded boxes, respectively. The mutated nucleotides of the Rp site are in boldfaced type. Luciferase activity was measured 72 h after transfection. RLU, relative light units; LUC, luciferase reporter gene.

However, a careful examination of the binding pattern of Rev-erbα on the WT73bp probe revealed the presence of a specific less intense complex of reduced mobility (arrowhead in Fig. 5*A Left*). To investigate whether this complex could correspond to a homodimer, we constructed a carboxyl-terminal truncated version of Rev-erbα, called REV-ERBα-(1–236). This truncated Rev-erbα protein lacks the entire ligand-binding E domain but still contains the A and T boxes and a portion of the hinge region D domain. As shown in Fig. 5*A*, when the WT73bp probe was coincubated with a mixture of the full-length and the truncated Rev-erbα protein, a new complex appeared. This complex corresponded to a dimer formed between the full-length and the truncated version of Rev-erbα protein (the same pattern was obtained with another version of Rev-erbα deleted after residue 288; data not shown). These results strongly suggest that Rev-erbα is capable of forming homodimers.

We next investigated which sequence of the 73-bp fragment was involved in the binding of the second molecule of the dimer. Two degenerate AGGTCA motifs were found within this sequence, located 2 bp and 23 bp, respectively, downstream from the Rp site (underlined in Fig. 2) both in a direct orientation relative to it. As shown in Fig. 5*B*, an oligonucleotide encompassing the Rp site and the AGGTCA-like motif 2 bp downstream, hereafter referred to as RevDR2, was sufficient to allow the binding of Rev-erbα as a dimer. To further investigate the respective role of these two half-site motifs on Rev-erbα binding, we used two oligonucleotides mutated either in the Rp core sequence or in the 3′ motif (RevDR2M5′ and RevDR2M3′, respectively). Mutation of the 3′ site abolished the binding of the dimer but still allowed the monomer to bind with an affinity comparable to that observed on the RevDR2 wild-type probe (Fig. 5*B*). Conversely, mutation of the 5′ core sequence completely abolished the binding of Rev-erbα onto this probe, reminiscent of the result obtained with the Δ73bp probe (Fig. 5*A*).

Rev-erbα Repressing Activity Does Not Depend on Its Homodimerization but Requires Its Carboxyl-Terminal Domain. To investigate whether homodimer formation was required for the repressing activity of Rev-erbα, two copies of either RevDR2, RevDR2M5′, or RevDR2M3′ oligonucleotides were cloned upstream of the SV40 early promoter. As shown in Fig. 5*C*, a 4-fold repression was observed upon cotransfection of pRevDR2-SV40 with the Rev-erbα expression vector. Conversely, no repression was seen when the reporter vector bore two copies of the responsive element mutated in its 5′ motif (pRevDR2M5′-SV40) or when the native SV40 reporter vector was used. Interestingly, a reporter

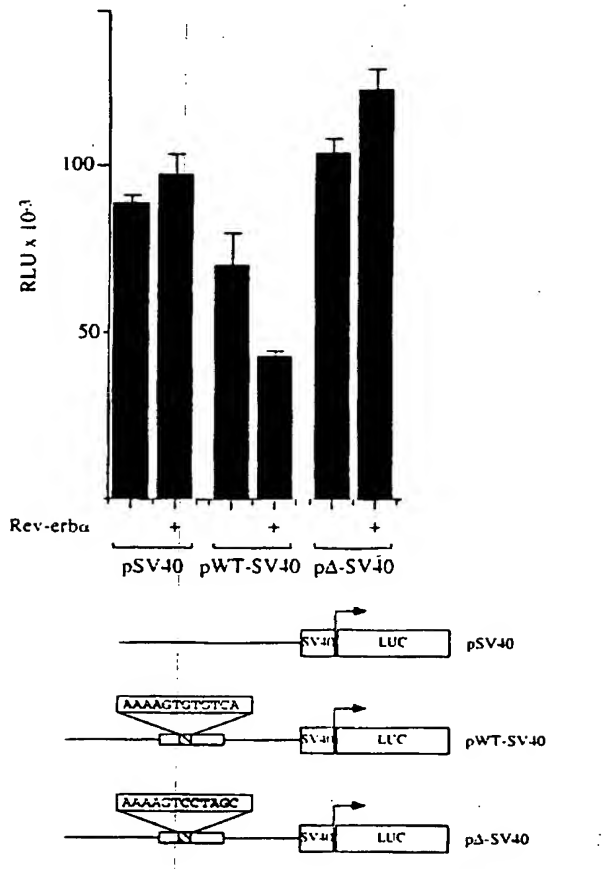

FIG. 4. Rev-erbα repression of the 73-bp *Sst* I–*Kpn* I promoter fragment placed upstream of the SV40 promoter. One microgram of the pSG5 or pREVERBα expression vector and 4 μg of each of the SV40 promoter constructs were cotransfected in HepG2 cells. Luciferase activity was measured 72 h after transfection. The 73-bp *Sst* I–*Kpn* I promoter fragment is shown as a stippled box. The hatched box represents the Rp site. Its mutated nucleotides are in boldfaced type. RLU, relative light units; LUC, luciferase reporter gene.

vector harboring two copies of the RevDR2M3′ oligonucleotide was still repressed by Rev-erbα. These results suggest that homodimer formation is dispensable for Rev-erbα repression.

To further support the evidence that the Rev-erbα repressing activity is an intrinsic function of the protein, we tested the effect of a truncated version of Rev-erbα that binds to the RevRE but that is deleted of its carboxyl-terminal moiety (see *Materials and Methods*). As shown in Fig. 5*C*, this truncated version of Rev-erbα, unlike the full-length protein, was unable to repress the activity of the pRevDR2-SV40 reporter. This result indicates that transcriptional repression is an intrinsic property of Rev-erbα and that this function is carried out by its carboxyl-terminal domain.

DISCUSSION

This work reports the characterization of a functional promoter for the human Rev-erbα gene. The analysis of the human Rev-erbα gene promoter is of interest in the context of the c-erbA-1/Rev-erbα overlapping locus, which encodes two related transcription factors. We and others (19, 23) have previously shown that the c-erbA-1 promoter responds to neither c-erbA-1 nor to Rev-erbα proteins. The availability of the Rev-erbα gene promoter will help us to decipher further the functional implications of these findings and particularly to investigate the potential autoregulation that takes place at this

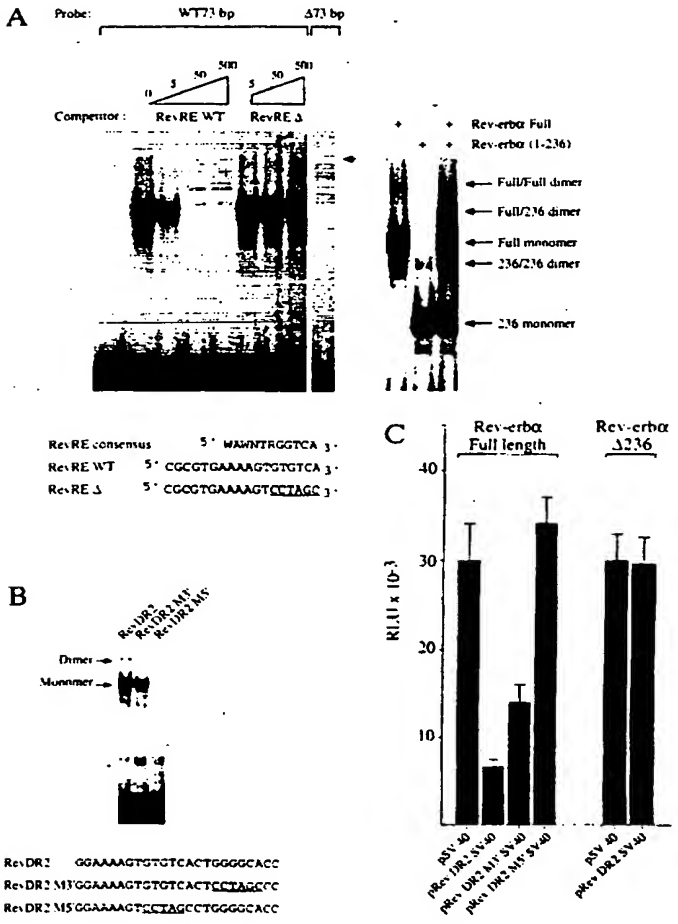

FIG. 5. (*A*) Rev-erbα binding to the 73-bp probe. (*Left*) One microliter of pSG5 control lysate (lane at the extreme left) or pREVERBα protein was incubated with 0.5 ng of the 73-bp labeled probe in its wild-type (WT73bp) or mutated version (Δ73bp). Competition experiments were carried out with a 5-, 50-, or 500-fold molar excess of either the synthetic RevREWT or the RevREΔ oligonucleotides as indicated. The arrowhead indicates the homodimer. The sequences of the competitor oligonucleotides are shown below the figure (mutated nucleotides are underlined). The RevRE consensus sequence is shown for comparison (W is A or T, N is any of the four bases, and R is A or G). (*Right*) Rev-erbα is able to bind the WT73bp probe as a homodimer. The 73-bp probe (0.5 ng) was incubated with 4 μl of the full-length REV-ERBα *in vitro*-translated protein, 1 μl of REV-ERBα-(1–236) *in vitro*-translated protein, or both as indicated. DNA–protein complexes are indicated at the right. The asterisk indicates an artifact that is likely to reflect the use of a downstream AUG. (*B*) Rev-erbα homodimer binds to the RevDR2 motif. Four microliters of the full-length REV-ERBα *in vitro*-translated protein was incubated with 0.5 ng of either RevDR2, RevDR2M3′, or RevDR2M5′ probes as indicated. The asterisk indicates a nonspecific complex. (*C*) Rev-erbα represses transcription via the 5′ motif of the RevDR2. HepG2 cells were cotransfected along with 3 μg of the pSVRev-erbα (Full length) or pSVRev-erbα (Δ236) expression vectors and 1 μg of the indicated reporter constructs. Luciferase activity was measured after transfection.

locus. It will also allow us to study the factors regulexhibiting expression during various physiological processes (In *in vitro*.

Recent investigations about the function of Rev the TrfA shown that, similar to other orphan receptors, Re2 origin and as a monomer to an AGGTCA motif preceded M three mutant sequence (10, 14). These authors also describ at the origin but constitutive transcription factor when boun mutant) or exhibbut others failed to reproduce this finding (discrepancies have been explained by work. unidentified serum factor inducing Ressed.

function, this hypothesis appears very unlikely since Rev-erbα lacks the crucial and strongly conserved ligand-regulated transactivating domain AF2 (24–26). As discussed in Durand *et al.* (25), it is more likely that orphan receptors lacking this AF2 domain would act as competitors for other ligand-inducible receptors. This is consistent with the recent findings that Rev-erbα passively blocks RZRα-mediated transactivation (15–17). While our study also reports that Rev-erbα mediates transcriptional repression, two lines of evidence argue for an intrinsic repressing effect of Rev-erbα. First, mutation of the Rp site did not decrease the basal activity of the Rev-erbα promoter, a consequence that would be expected if endogenous RZRα transactivated through this sequence. Second, a truncated version of Rev-erbα that still binds DNA was no longer able to repress transcription. This latter result evidenced the intrinsic repressive property of Rev-erbα. It remains to be investigated whether some elements, such as the binding site sequence or a protein partner, induce Rev-erbα to act as a passive competitor or as an "active" repressor.

Our study also reports that Rev-erbα is able to bind DNA as a homodimer. This homodimer binds an asymmetric RevDR2 element in which the upstream motif is extended with a 5′ A/T-rich sequence. This asymmetric interaction between two Rev-erbα monomers bound to a direct repeat element is reminiscent of what was recently described for HNF4 bound to a DR1 (29). Nevertheless, as for the orphan receptor RZRα, Rev-erbα homodimerization does not seem as favored as in the case of retinoic acid receptor or thyroid hormone receptor homodimer formation (27). We also show that, while Rev-erb efficiently binds the isolated 5′ binding site, its binding to the 3′ site was abolished when the 5′ sequence was mutated. Similarly, although the RevDR2 5′ site is necessary and sufficient to mediate the Rev-erbα repression, the 3′ motif of RevDR2 is not. The ability of Rev-erbα to dimerize is an interesting feature, but its function remains to be established.

The ability to form homodimers sheds new light on the diversification of the binding capacities of nuclear receptors during evolution (10). Based on the description of an increasing number of nonevolutionarily linked orphan receptors that are able to bind DNA exclusively as monomers, some authors have hypothesized that the ancestral nuclear orphan receptor bound DNA as a monomer (28). The fact that Rev-erbs as well as RZRs (27) are able to homodimerize allows us to consider that binding as monomers was gained independently by some orphan receptors during evolution (10). These orphan receptors have not necessarily lost their ability to efficiently homo- or heterodimerize, as is the case for RZR and Rev-erbα.

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General information

Entry name	**NRD1_HUMAN**
Accession number	**P20393**, Q15304
Created	Rel. 17, 1-FEB-1991
Sequence update	Rel. 17, 1-FEB-1991
Annotation update	Rel. 41, 15-JUN-2002

XP-002226720

P.D. 01-02-1991 3
P. 1 - 3

Description and origin of the Protein

Description	Orphan nuclear receptor NR1D1 (V-erbA related protein EAR-1) (Rev- erbA-alpha).
Gene name(s)	NR1D1 OR THRAL OR EAR1 OR HREV.
Organism source	Homo sapiens (Human).
Taxonomy	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
NCBI TaxID	9606

References

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Two erbA homologs encoding proteins with different T3 binding capacities are transcribed from opposite DNA strands of the same genetic locus.
(1989) *Cell* **57**:31-39

Position	SEQUENCE FROM N.A., FUNCTION, AND TISSUE SPECIFICITY.
Comments	TISSUE=PLACENTA;
Medline	89195219
PubMed	2539258

[2] Lazar,M.A., Jones,K.E., Chin,W.W.,
Isolation of a cDNA encoding human Rev-ErbA alpha: transcription from the noncoding DNA strand of a thyroid hormone receptor gene results in a related protein that does not bind thyroid hormone.
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Position	SEQUENCE FROM N.A., AND FUNCTION.
Comments	TISSUE=FETAL SKELETAL MUSCLE;
Medline	90262650
PubMed	1971514

[3] Laudet,V., Begue,A., Henry,C., Joubel,A., Martin,P., Stehelin,D., Saule,S.,
Genomic organization of the human thyroid hormone receptor alpha (c- erbA-1) gene.
(1991) *Nucleic Acids Res.* **19**:1105-1112

Position	SEQUENCE OF 549-614 FROM N.A.
Medline	91212192
PubMed	1850510

[4] Zhao,Q., Khorasanizadeh,S., Miyoshi,Y., Lazar,M.A., Rastinejad,F.,
Structural elements of an orphan nuclear receptor-DNA complex.
(1998) *Mol. Cell* **1**:849-861

Position	X-RAY CRYSTALLOGRAPHY (2.3 ANGSTROMS) OF 123-215.
Medline	98325398
PubMed	9660968

Comments

FUNCTION	POSSIBLE RECEPTOR FOR TRIIODOTHYRONINE.
SUBCELLULAR LOCATION	NUCLEAR (POTENTIAL).
TISSUE SPECIFICITY	EXPRESSED IN ALL TISSUES AND CELL LINES EXAMINED. EXPRESSED AT HIGH LEVELS IN SOME SQUAMOUS CARCINOMA CELL LINES.
DOMAIN	COMPOSED OF THREE DOMAINS: A MODULATING N-TERMINAL DOMAIN, A DNA-BINDING DOMAIN AND A C-TERMINAL STEROID-BINDING DOMAIN.
SIMILARITY	BELONGS TO THE NUCLEAR HORMONE RECEPTOR FAMILY. NR1 SUBFAMILY.

Copyright

Database cross-references

EMBL	M24898;AAA52335.1;-.
	M24900;AAA52332.1;-.
	X55066;-;NOT_ANNOTATED_CDS.
	X55067;-;NOT_ANNOTATED_CDS.
	X72631;CAB53540.1;-.
	M34339;AAA36561.1;-.
	M34340;AAA36562.2;-.
PIR	A32286;A32286.
PDB	1A6Y;21-OCT-98.
TRANSFAC	T02746;-.
Genew	HGNC:7962;NR1D1.
MIM	602408;-.
InterPro	IPR000536;Hormone_rec_lig.
	IPR001723;Stdhrmn_receptor.
	IPR001628;Znf_C4steroid.
Pfam	PF00104;hormone_rec;1.
	PF00105;zf-C4;1.
PRINTS	PR00398;STRDHORMONER.
	PR00047;STROIDFINGER.
ProDom	PD000035;Znf_C4steroid;1.
SMART	SM00430;HOLI;1.
	SM00399;ZnF_C4;1.
PROSITE	PS00031;NUCLEAR_RECEPTOR;1.

Keywords

Receptor; Transcription regulation; DNA-binding; Nuclear protein; Zinc-finger; 3D-structure;

Features

Key	Begin	End	Length	Description
DOMAIN	82	93	12	POLY-SER.
DNA_BIND	132	198	67	NUCLEAR RECEPTOR-TYPE.
ZN_FING	132	152	21	C4-TYPE.
ZN_FING	169	193	25	C4-TYPE.
CONFLICT	147	147	1	H -> L (IN REF. 2).
CONFLICT	564	564	1	E -> Q (IN REF. 2).
STRAND	131	131	1	
TURN	133	135	3	
STRAND	138	138	1	
STRAND	143	143	1	
TURN	144	145	2	
STRAND	146	146	1	
HELIX	150	160	11	
TURN	161	161	1	
TURN	170	171	2	
TURN	179	184	6	
HELIX	186	196	11	
TURN	197	197	1	
TURN	200	201	2	

Sequence information

Length: **614 aa**, molecular weight: **66805 Da**, CRC64 checksum: **67C71758E166508A**

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NITKLNGMVL LCKVCGDVAS GFHYGVHACE GCKGFFRRSI QQNIQYKRCL KNENCSIVRI     180
NRNRCQQCRF KKCLSVGMSR DAVRFGRIPK REKQRMLAEM QSAMNLANNQ LSSQCPLETS     240
PTQHPTPGPM GPSPPPAPVP SPLVGFSQFP QQLTPPRSPS PEPTVEDVIS QVARAHREIF     300
TYAHDKLGSS PGNFNANHAS GSPPATTPHR WENQGCPPAP NDNNTLAAQR HNEALNGLRQ     360
APSSYPPTWP PGPAHHSCHQ SNSNGHRLCP THVYAAPEGK APANSPRQGN SKNVLLACPM     420
NMYPHGRSGR TVQEIWEDFS MSFTPAVREV VEFAKHIPGF RDLSQHDQVT LLKAGTFEVL     480
MVRFASLFNV KDQTVMFLSR TTYSLQELGA MGMGDLLSAM FDFSEKLNSL ALTEEELGLF     540
TAVVLVSADR SGMENSASVE QLQETLLRAL RALVLKNRPL ETSRFTKLLL KLPDLRTLNN     600
MHSEKLLSFR VDAQ                                                       614
//
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General | Description | References | Comments | Links | Keywords | Features | Sequence

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Induction of Rev-ErbAα, an Orphan Receptor Encoded on the Opposite Strand of the α-Thyroid Hormone Receptor Gene, during Adipocyte Differentiation*

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P16265·16269

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Rev-ErbAα (Rev-Erb) is a nuclear hormone receptor-related transcriptional activator that is encoded on the noncoding strand of the α-thyroid hormone receptor (TR) gene. The similarities between Rev-Erb and receptors for differentiating agents, as well as the abundance of Rev-Erb mRNA in fat, led us to study Rev-Erb gene expression during adipogenesis. Remarkably, Rev-Erb mRNA levels increased dramatically during the differentiation of 3T3-L1 cells into adipocytes. Rev-Erb was similarly induced in the related 3T3-F442A cell line but not in nondifferentiating 3T3-C2 cells. The time course of Rev-Erb induction was similar to that of C/EBPα, an important transcriptional regulator in adipocytes, and Rev-Erb mRNA was superinduced by cycloheximide. Nuclear run-on assays indicated that an increased rate of Rev-Erb mRNA synthesis accounted for the increased steady state mRNA levels; the half-life of Rev-Erb mRNA was indistinguishable in preadipocytes and adipocytes. Treatment of preadipocytes with retinoic acid inhibited adipocyte differentiation and also prevented Rev-Erb induction. Thus, there is a correlation between Rev-Erb gene expression and differentiation, and transcriptional regulation by Rev-Erb could play an important role in the generation and/or maintenance of the adipocyte phenotype. Interestingly, and possibly related to the overlap between the Rev-Erb gene and the exon specific for TRα2, the induction of Rev-Erb was also associated with a 3-fold increase in the ratio of TRα1 to TRα2 mRNA levels, indicating that Rev-Erb expression has the potential to modulate adipocyte gene expression by multiple mechanisms.

Rev-ErbAα (Rev-Erb,[1] also called *ear 1*) is a member of the steroid/thyroid hormone receptor superfamily (1, 2) with considerable homology to receptors for thyroid hormone (T3) and retinoic acid (RA) (3, 4). Remarkably, both the rat and human Rev-Erb genes are transcribed from the opposite strand of the T3 receptor α gene, which itself encodes TRα1 and the splice variant TRα2 (5–9), the latter of which is a non-T3-binding inhibitor of T3 action (10, 11). The Rev-Erb and TRα2 genes actually overlap, and their mRNA products are complementary over a stretch of 269 nucleotides. Rev-Erb induction correlates with an increased ratio of TRα1 to TRα2 mRNA (12), and a causal relationship between these observations is supported by the observation that Rev-Erb mRNA can inhibit the splicing reaction that generates TRα2 *in vitro* (13).

We have recently demonstrated that the Rev-Erb binds with high affinity to a unique DNA sequence and is capable of activating gene transcription from this specific response element in the absence of exogenous ligand (14). However, although Rev-Erb gene expression is tissue-specific (3) and is regulated at transcriptional and post-transcriptional levels by labile proteins in a variety of cell types (12), the biological function of Rev-Erb is unknown.

A number of the receptors that are homologous to Rev-Erb are involved in cellular differentiation. Indeed, of all the members of the steroid/thyroid hormone receptor superfamily, Rev-Erb is most similar to E75, an ecdysone-induced transcription factor involved in the metamorphosis of *Drosophila melanogaster* (15). Because of this, we hypothesized that Rev-Erb may be involved in the differentiation of fat, where its mRNA is highly expressed (3). Indeed, Rev-Erb mRNA levels increased dramatically during the differentiation of 3T3-L1 and 3T3-F442A preadipocytes into adipocytes. This induction occurred at the level of gene transcription and was blocked by RA, which inhibits adipocyte differentiation (16, 17). The induction of Rev-Erb was accompanied by an increased ratio of TRα1 to TRα2. Altered levels of Rev-Erb and TRs could have important effects on the adipocyte phenotype.

MATERIALS AND METHODS

Cell Culture and Differentiation—3T3-L1 cells (obtained from the ATCC) were cultured in growth medium containing Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) and 10% bovine calf serum (Hyclone), with a change of media every 2 days. The cells were differentiated by the method of Bernlohr *et al.* (18). 2 days post-confluent cells (designated day 0) were switched to differentiation medium (DMEM, 10% fetal calf serum (Hyclone), 1 μM dexamethasone, 10 μg/ml insulin, and 0.5 mM 3-methyl-1-isobutylxanthine (Sigma)) for 2 days. Thereafter, the cells were cultured in post-differentiation medium (DMEM, 10% fetal calf serum, and 10 μg/ml insulin), and the media was changed every 2 days. Differentiation was maximal (~90% of the cells) by day 7, using morphological criteria. The 3T3-F422A cells (provided by Dr. V. Cherington, Tufts University, Boston) were cultured in the growth medium with a change of media every 2 days, and differentiation was induced in confluent plates (designated day 0) by changing the media to DMEM supplemented with 10% fetal calf serum and 10 μg/ml insulin (19).

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‡ Supported by the Medical Scientist Training Program.

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[1] The abbreviations used are: Rev-Erb, Rev-ErbAα, also called *ear 1*; TR, α-thyroid hormone receptor; RA, retinoic acid; DMEM, Dulbecco's modified Eagle's medium.

Cells were maintained in this medium, with a change every 2 days, until processed for RNA on day 18, when maximal differentiation was achieved. The 3T3-C2 cells (obtained from Dr. V. Cherington) were cultured in a manner similar to 3T3-L1 cells.

To determine Rev-Erb mRNA half-life in preadipocytes and adipocytes, actinomycin D (5 μg/ml) was added to the culture medium of days 0 and 7 cells for varying times prior to isolation of RNA. For determination of the mechanism of Rev-Erb mRNA induction, days 0 and 7 cells were treated with cycloheximide (10 μg/ml) for 20 h prior to isolation of RNA. For inhibition of differentiation by all-*trans* RA, 10 μM RA (in ethanol) was added to day 0 3T3-L1 cells at the same time as differentiation medium. This concentration of RA completely inhibited adipose conversion, as previously shown (20). After 48 h, the media was changed to post-differentiation medium alone. All control cultures were treated with an equal volume of ethanol.

Northern Analysis—Northern analyses were performed on total celluar RNA as described previously using cDNA probes for Rev-Erb (3), C/EBPα (21), aP2 (22), and β-actin labeled with ^{32}P using random primers (Boehringer Mannheim). TRα1 and TRα2 mRNA were both detected using a TRα riboprobe (23). Autoradiographs were quantitated using a Molecular Dynamics ImageQuant densitometer. Statistical analyses were performed using Student's *t* test (Statworks program), and linear regressions for mRNA half-life studies were done using Sigma Plot (Jandel Scientific).

Nuclear Transcription Rate Assays—Cells were pooled from four 15-cm dishes of preadipocytes and adipocytes, and nuclei were isolated in Nonidet P-40 lysis buffer (10 mM Tris·HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% (v/v) Nonidet P-40) by the method of Greenberg and Ziff (24). Transcription reactions were done as described by Distel *et al.* (25). Briefly, transcription was initiated by the addition of equal volumes of 2 × reaction buffer (10 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 5.0 mM dithiothreitol, 80 units RNasin (Promega), 1.0 mM dNTPs, and 200 μCi [^{32}P]UTP (800 Ci/mmol, Amersham Corp.)) to 2–4 × 10^7 nuclei (in 150–200 μl). Reaction mixture was incubated at 30 °C for 30 min with gentle agitation. Slot blots were prepared by applying 10 μg of denatured cDNAs to Duralon membrane (Stratagene) as per the manufacturer's protocol. Nascent transcripts were isolated as previously described (23) and hybridized with the slot blots at 42 °C for 4 days. Slot blots were washed twice at 55 °C for 45 min in 0.3 × SSC, 0.1% SDS, autoradiographed, and quantitated densitometrically.

RESULTS

Rev-Erb Is Induced during Adipogenesis—3T3-L1 preadipocytes were grown to confluence and then switched to differentiation conditions 2 days later as described under "Materials and Methods." After 7 days, ~90% of the cells were rounder and loaded with fat, consistent with differentiation into adipocytes. Fig. 1 shows that expression of the ~3.0-kb Rev-Erb mRNA was 12.8-fold greater in adipocytes than in preadipocytes. This difference was not unique to 3T3-L1 cells, because similar changes were also noted after differentiation of another adipogenic cell line, 3T3-F442A. The effect was specific for adipocytes, however, because Rev-Erb mRNA levels actually decreased when 3T3-C2 cells (a related, nondifferentiating cell line) (25, 26) were exposed to the same differentiating conditions as 3T3-L1 cells. The time course of Rev-Erb induction in 3T3-L1 cells is shown in Fig. 2. Rev-Erb was half-maximally induced by day 3 and maximally induced by day 5. This pattern of gene expression was compared with those of other genes previously shown to be induced during adipogenesis. Induction of the leucine zipper-containing transcription factor C/EBPα occurred about the same time and was of similar magnitude as that of Rev-Erb, while induction of the fat-specific gene aP2 was of much greater magnitude. β-actin mRNA levels showed little change during adipogenesis.

Rev-Erb Induction Is Transcriptional—The mechanism of Rev-Erb induction in 3T3-L1 cells was investigated next. Nuclear run-on assays were performed using nuclei from preadipocytes and adipocytes to determine whether there was

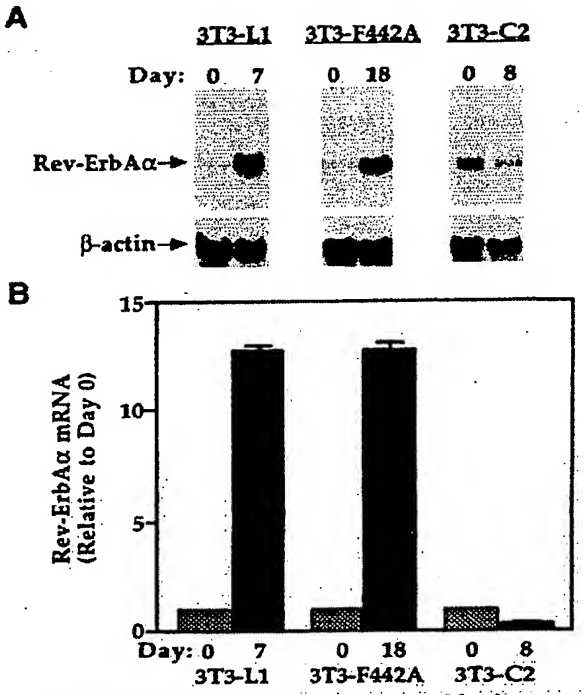

FIG. 1. **Rev-Erb induction in adipocytes.** *A*, Northern analysis of 3T3-L1, 3T3-F442A, and 3T3-C2 cells before and after exposure to differentiating conditions. The duration of treatment was different for the three cell lines (7, 18, and 8 days, respectively; see "Materials and Methods"). β-actin mRNA is shown as control. Equal loading was confirmed by ethidium bromide fluorescence of 28 S and 18 S rRNA, which varied insignificantly from lane to lane. *B*, quantitation of changes in Rev-Erb mRNA in multiple experiments (3T3-L1, $n = 14$; 3T3-F442A, $n = 2$; 3T3-C2, $n = 3$), expressed in arbitrary densitometric units.

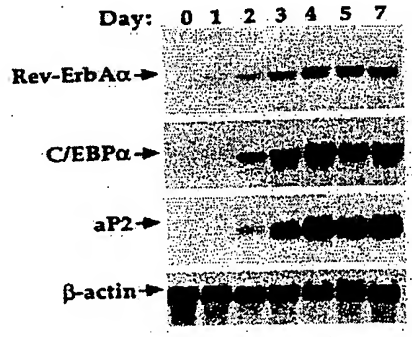

FIG. 2. **Time course of Rev-Erb induction.** Levels of mRNAs encoding Rev-Erb, C/EBPα, aP2, and β-actin were assayed at varying times after exposure of 3T3-L1 cells to differentiating conditions (see "Materials and Methods"). Ethidium bromide fluorescence of 28 S and 18 S rRNA varied insignificantly from lane to lane. β-actin mRNA is shown as control.

an increase in the rate of Rev-Erb gene transcription. Fig. 3*A* shows that Rev-Erb transcription is increased in adipocytes, with a mean increase in seven separate experiments of 12.4-fold, similar to the increase in the steady state mRNA level (Fig. 3*B*). The transcription of C/EBPα also increased in parallel with its mRNA level, while the rate of β-actin gene transcription did not change greatly, in agreement with the Northern results shown earlier. The transcriptional rate of aP2 was also increased, although the magnitude of the change was less than that of aP2 mRNA, consistent with post-transcriptional regulation as observed by others (19, 27).

Rev-Erb mRNA Is Not Stabilized in Adipocytes—Although

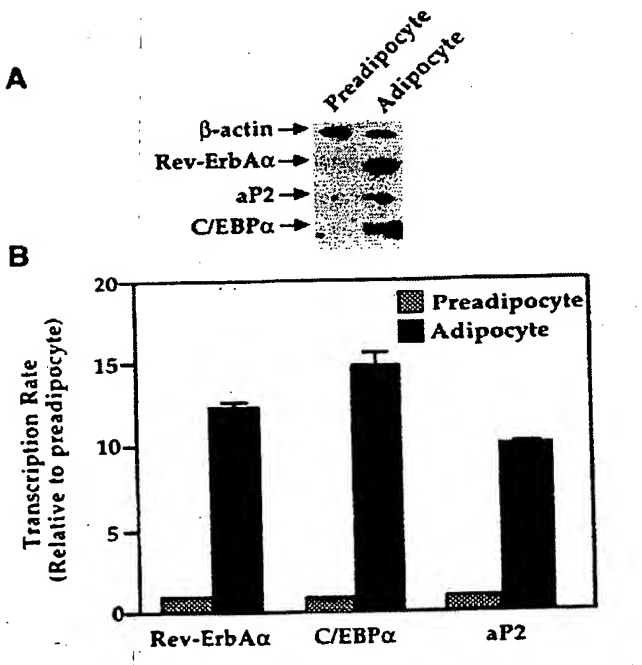

FIG. 3. **Increased transcription of Rev-Erb in adipocytes.** *A*, transcriptional rate assays were performed with nuclei from 3T3-L1 preadipocytes and adipocytes. The relative transcription rates of β-actin, Rev-Erb, aP2, and C/EBPα were compared. *B*, densitometric quantitation of the changes in transcription in multiple experiments (Rev-Erb, $n = 7$; C/EBPα, $n = 7$; aP2, $n = 2$. Results were normalized to β-actin and then compared with preadipocyte levels and expressed in arbitrary densitometric units.

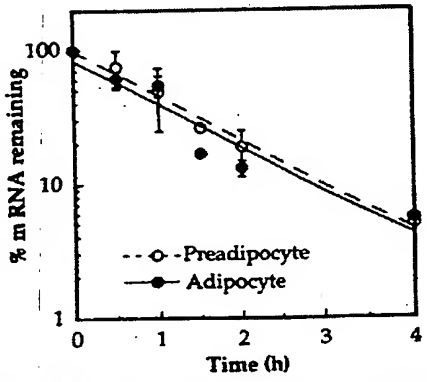

FIG. 4. **Half-life of Rev-Erb mRNA in preadipocytes and adipocytes.** Cells were treated with actinomycin D (see "Materials and Methods") prior to RNA preparation at the indicated times. Mean ± S.E. of four experiments are expressed relative to baseline Rev-Erb mRNA levels measured in arbitrary densitometric units. Results in preadipocytes are plotted (- -O- -), and results in adipocytes are shown (–●–).

the increase in Rev-Erb transcription rate was a sufficient explanation for the increase in steady state mRNA levels, we were nevertheless interested in the stability of the Rev-Erb mRNA, because we have previously shown that levels of Rev-Erb mRNA are regulated post-transcriptionally under some circumstances (12), and other mRNAs, such as that for aP2, are stabilized in adipocytes (19). Therefore, preadipocytes and adipocytes were treated with actinomycin D to inhibit new mRNA synthesis, and the rate of decay of Rev-Erb mRNA was assessed. Fig. 4 shows that the half-life of Rev-Erb mRNA was ~1 h in both preadipocytes and adipocytes, suggesting that Rev-Erb induction in adipocytes is due entirely to a transcriptional mechanism.

Rev-Erb Is Superinduced by Cycloheximide—We have previously shown that cycloheximide treatment of a variety of cell lines resulted in induction of Rev-Erb mRNA by a great increase in mRNA stability coupled with an increase in Rev-Erb gene transcription, which is of the same magnitude as that observed in the course of adipogenesis (12). We were therefore interested in whether the same mechanism is responsible for induction of Rev-Erb during cycloheximide treatment and adipose conversion. Fig. 5 shows that the low basal level of Rev-Erb expression in 3T3-L1 preadipocytes was greatly enhanced (~100-fold) by inhibition of protein synthesis by cycloheximide; as expected, the induction of Rev-Erb during differentiation was less (~10-fold), since no mRNA stabilization is involved, as shown earlier. Of greater interest was the observation that cycloheximide superinduced Rev-Erb expression in adipocytes. Indeed, the level of Rev-Erb expression under these circumstances was greater than that seen after cycloheximide treatment of preadipocytes, indicating that mechanism of Rev-Erb induction during adipogenesis is likely to be different than the mechanism of Rev-Erb induction by inhibition of protein synthesis. Whether induction of Rev-Erb during differentiation is due to the loss of a repressor or the gain of a transcriptional activator remains to be established.

Rev-Erb Induction Is Blocked by Retinoic Acid—RA, which acts by receptors that are structurally similar to Rev-Erb (1, 2), has been shown to inhibit differentiation of 3T3-L1 cells (20). Fig. 6 shows that simultaneous treatment of preadipocytes with RA and differentiating conditions completely inhibited the induction of Rev-Erb, which was observed after

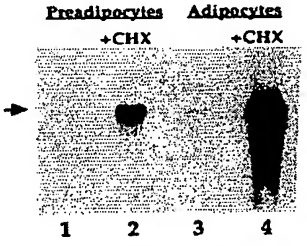

FIG. 5. **Superinduction of Rev-Erb mRNA.** Preadipocytes and adipocytes were treated (*lanes 2* and *4*) and without (*lanes 1* and *3*) cycloheximide (*CHX*) for a 20-h prior to RNA preparation and Northern analysis. *Arrow* points to Rev-Erb mRNA.

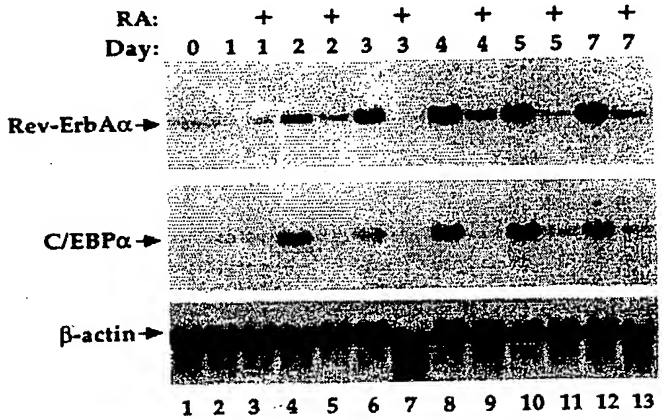

FIG. 6. **Induction of Rev-Erb is inhibited by RA.** Preadipocytes were treated in the same way as in the experiment shown in Fig. 2, except that 10 μM RA was added to parallel plates at day 0 for each time point. Ethidium bromide fluorescence of 28 S and 18 S rRNA varied insignificantly except in *lane 7*, where the RNA was somewhat degraded.

exposure to differentiating conditions in the absence of RA. This result suggests that increased Rev-Erb gene expression is truly part of the differentiation program and unlikely to be due simply to a specific effect of one of the differentiating agents. Similarly, Rev-Erb is only slightly induced by single agents, such as dexamethasone in the absence of fetal calf serum, which do not result in adipocyte conversion (data not shown).

Rev-Erb Induction Correlates with Increased TRα1/TRα2 mRNA Ratio—Since Rev-Erb mRNA is complementary to that of TRα2 and Rev-Erb gene expression has been shown to correlate with the ratio of TRα1 to TRα2 mRNAs in some circumstances (12), we also studied TRα gene expression in preadipocytes and adipocytes. As shown in Fig. 7*A*, levels of TRα1 mRNA increased during the differentiation of 3T3-L1 cells, while TRα2 levels dropped relative to the level in preadipocytes. Quantitation of the results of ten experiments (Fig. 7*B*) indicated that TRα mRNA increased an average of 1.5-fold, and the mean decrease in TRα2 mRNA was 45%. Thus, the ratio of TRα1 to TRα2 increased by an average of 3-fold. The TRα1/TRα2 mRNA ratio is a function of the processing of the TRα gene transcript as well as the half-lives of the individual TRs. There was no significant difference between preadipocyte and adipocyte half-lives of both the TRα1 and TRα2 mRNAs (data not shown), although the half-life of TRα1 was considerably shorter than that of TRα2 mRNA, as was also observed in rat 235–1 cells (12). These data suggest that the change in α1/α2 ratio during differentiation was due to a change in the alternative splicing/polyadenylation of the TRα pre-mRNA.

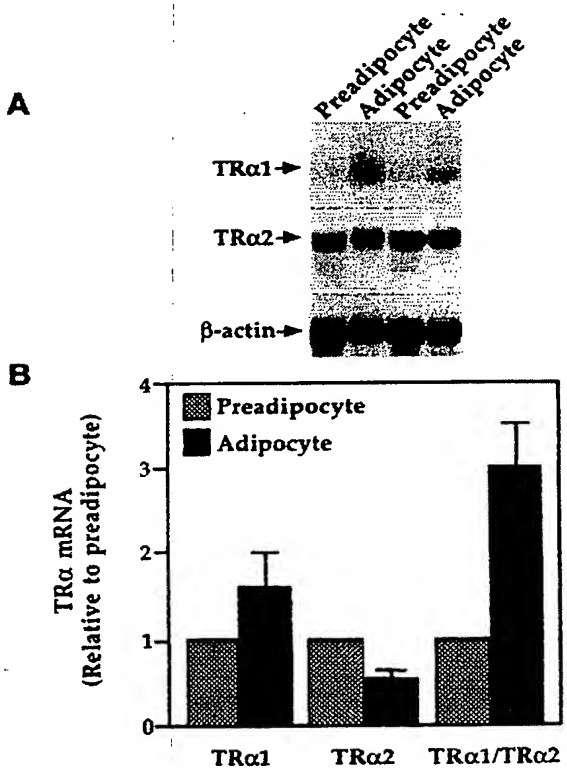

FIG. 7. **TR α mRNA levels in preadipocytes and adipocytes.** *A*, Northern analysis of TRα1 and TRα2 mRNA. The identical probe was used to detect TRα1 and TRα2, but the autoradiographic exposure time of the TRα2 signal was 6 h, while that for TRα1 was 14 h. β-actin mRNA is shown as control. *B*, densitometric quantitation of the results of ten separate experiments. Results are expressed relative to base-line mRNA levels. For TRα1 and TRα2 mRNA, units are arbitrary densitometric units. There are no units for the TRα1/TRα2 mRNA ratio.

DISCUSSION

We have used the model of adipose conversion of 3T3 preadipocytes (26, 28, 29) to show that Rev-Erb, a transcription factor similar in structure to nuclear hormone receptors, is dramatically induced during adipogenesis. It is interesting that Rev-Erb is most highly related to E75, a protein that is induced early in molting of *D. melanogaster* (15), and is also very much like receptors for thyroid hormone, RA, and vitamin D, all of which are involved in various aspects of vertebrate differentiation (30). Rev-Erb could be a primary mediator of adipogenesis, either unliganded or in response to a factor present either in serum or in the cell. Alternatively, Rev-Erb may be involved in maintenance of the adipocyte phenotype once transiently induced transcription factors, such as C/EBPβ and δ (31) as well as c-*fos* (32) and c-*jun* (20), have returned to basal levels. Indeed, the transcriptional activation of Rev-Erb may be a direct result of the increase in one or more of the primary responders to adipogenic conditions. Once induced, high levels of Rev-Erb could be maintained by direct positive feedback by Rev-Erb itself and/or by other transcription factors that remain abundant in differentiated adipocytes. C/EBPα may be of particular importance, since it induces mitotic arrest (33) and activates adipocyte-specific genes (34, 35), and its expression correlates with the adipocyte phenotype (36–39). These questions are currently being addressed in our laboratory.

The effects of RA on Rev-Erb gene expression could be direct or indirect, although the inhibition of other markers of the differentiated phenotype such as C/EBPα suggests that RA may act at an earlier step in adipogenesis. The inhibitory effect of RA on adipocyte differentiation is not well understood but is presumably mediated by RA receptors or by receptors for the RA isomer, 9-*cis* RA (40). Multiple RA receptors and 9-*cis* RA receptor subtypes are expressed in 3T3-L1 cells,[2] and both of these receptor types are closely related to Rev-Erb. 9-*cis* RA receptors in particular are known to be heterodimerization partners for a number of related receptors (see Ref. 40 and references therein). Thus, interactions between members of the thyroid/steroid hormone receptor superfamily could be very important during and after adipocyte differentiation.

The correlation between Rev-Erb gene expression and the ratio of TRα1 to TRα2 during adipogenesis is consistent with the hypothesis that Rev-Erb gene expression may regulate the alternative splicing of the TRα transcript (13). The magnitude of the change in α1/α2 mRNA ratio during adipogenesis is less than that observed after cycloheximide treatment (3), consistent with the more profound effect of cycloheximide on Rev-Erb gene expression (Fig. 5) (12). However, in the absence of cycloheximide, it is likely that the TR protein levels will change as well. The predicted increase in TRα1 and decrease in the T3-action inhibitor TRα2 would be expected to magnify the effects of T3 on gene expression in adipocytes, which have undetectable levels of TRβ mRNA by Northern analysis (41).[2] Indeed, T3 is a major regulator of thermogenesis (42) and has numerous effects on adipocytes in culture (43, 44) as well as on fat cells *in vivo* (45, 46). Taken together with the likely function of Rev-Erb itself as a transcriptional activator, these results suggest that Rev-Erb induction plays a major role in the regulation of adipocyte gene expression.

[2] A. Chawla and M. A. Lazar, unpublished data.

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(5)

Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival

(nuclear receptors/adipocytes/differentiation)

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ABSTRACT **Culture of mouse 3T3-L1 preadipocytes in medium containing delipidated bovine calf serum caused the cells to elongate and divide after reaching confluency. The continued proliferation correlated with sustained expression of the c-*myc* gene, which was repressed in control cells. Exposure of the cells to activators of peroxisome proliferator-activated receptor (PPAR), including clofibrate, WY-14,643, and 5,8,11,14-eicosatetraynoic acid, reversed and prevented the effects of culturing preadipocytes in delipidated serum. Continued exposure to PPAR activators led to adipose conversion, during which PPAR and its heterodimerization partner (retinoid X receptor) were induced. Retinoic acid (RA) had no effect on the growth or survival of preadipocytes grown in the presence of normal bovine serum. However, treatment of cells cultured in delipidated serum with RA caused death of the cells by apoptosis. Thus, preadipocyte phenotype and survival are regulated by activators of nuclear hormone receptors.**

3T3-L1 cells have been selected from Swiss 3T3 mouse fibroblasts for their ability to differentiate into adipocytes after exposure to fetal calf serum (FCS), dexamethasone (Dex), isobutylmethylxanthine (IBMX), and insulin (1, 2). Adipose conversion involves coordinated expression of a variety of transcription factors prior to the morphological changes and induction of structural proteins (3). Factors induced during adipocyte differentiation include C/EBPα, -β, and -δ (4, 5) as well as Jun and Fos (6, 7). In addition, c-*myc* gene expression is repressed in the adipocyte, and overexpression of c-*myc* can inhibit differentiation (8). However, the biological basis of the adipose commitment of the 3T3-L1 cell is not well understood.

Retinoic acid (RA) and peroxisome proliferators are lipophilic substances that act through related nuclear receptors to directly regulate gene expression (9–12). Both RA receptors (RARs) and peroxisome proliferator-activated receptors (PPARs) heterodimerize with retinoid X receptor (RXR) *in vitro* (ref. 12 and references therein) and, in the case of PPAR, regulation of gene transcription is greatest when the activators of both receptors are present (13–16). RA prevents the adipose conversion of 3T3-L1 cells (17, 18). Inhibition of differentiation is an unusual effect of RA, which more commonly promotes differentiation (19). In contrast to the effects of RA, peroxisome proliferators such as clofibrate have been shown to potentiate adipose conversion of 3T3-L1 cells in the presence of Dex, FCS, and insulin (20).

PPAR is of particular interest in adipocytes because it regulates genes involved in lipid metabolism (21, 22) and is activated not only by peroxisome proliferators but by fatty acids (13, 23) as well. We have found that culturing 3T3-L1 preadipocytes in medium containing delipidated serum induces a proliferative and elongated phenotype that is reversed by PPAR activators. Furthermore, a variety of PPAR activators cause adipose conversion in the absence of Dex, IBMX, FCS, and insulin. PPARs and RXRs are both induced during adipose conversion, which is inhibited by RA. However, exposure of the cells cultured in delipidated medium to RA results in cell death by apoptosis. Thus, PPAR activators and retinoids co-regulate preadipocyte proliferation, differentiation, and survival.

MATERIALS AND METHODS

Cell Culture. 3T3-L1 cells (ATCC) were cultured in growth medium containing Dulbecco's modified Eagle's medium and 10% bovine calf serum (HyClone) with a change of medium every 2 days. Bovine calf serum was delipidated by a modification of the method of Goodman (24). Briefly, serum was extracted two or three times with an equal volume of *n*-heptane by vigorous stirring at 4°C for 20 h. The aqueous phase was separated by centrifugation at 2000 × *g* for 2 h. Cells were initially cultured in normal growth medium for 2–3 days (40–50% confluence) and then switched to medium containing 10% delipidated serum.

The standard method of cell differentiation and inhibition by RA was as described (25). RA was added in ethanol at a final concentration of 10 μM. For experiments with PPAR activators, 3T3-L1 cells were cultured in growth medium until confluent and then switched to growth medium supplemented with various concentrations of clofibrate, WY-14,643 (pirinixic acid; kindly supplied by Wyeth–Ayerst), or 5,8,11,14-eicosatetraynoic acid (ETYA) in ethanol. Control cells were treated with the same volume of ethanol alone.

Northern Blot Analysis. Northern blot analyses were performed as described (25) with cDNA probes for aP2 (26); C/EBPα (27); PPARα (11); RXRα, -β, and -γ (28); Nuc-1 (29); c-myc; and β-actin labeled with ^{32}P using random hexamers.

Cell Viability. At 40–50% confluence, cells cultured in 60-mm^2 dishes were switched from control to delipidated medium containing either 10 μM RA or ethanol alone. Approximately every 24 h, floating and adherent cells were pooled and viability was assessed by trypan blue exclusion. Similar results were observed when cells were exposed to RA throughout the experiment or only during the initial 48 h. For determination of [^{3}H]thymidine incorporation, cells were cultured in 96-well plates and [^{3}H]thymidine (5 μCi per well; 1 Ci = 37 GBq) was added for 1 h, after which the cells were harvested and incorporated radioactivity was measured by scintillation counting. Genomic DNA was isolated from

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Abbreviations: FCS, fetal calf serum; Dex, dexamethasone; IBMX, isobutylmethylxanthine; RA, retinoic acid; RAR, RA receptor; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; ETYA, 5,8,11,14-eicosatetraynoic acid.

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adherent and floating cells by a method that selected for fragmented DNA (30) and was analyzed in 1.2% agarose gels.

RESULTS

Characteristics of Preadipocytes Grown in Delipidated Serum. Preadipocytes were cultured in medium containing bovine calf serum, which was extracted with *n*-heptane to remove lipophilic substances, including fatty acids and retinoids (24). Under these conditions, preadipocytes continued to proliferate after control cells had reached confluence, and the number of cells was increased ≈3-fold after 4 days (Fig. 1*A*). [^{3}H]Thymidine incorporation of the cells in delipidated serum was ≈5-fold greater than that of control cells, which decreased by 88% after confluence (Fig. 1*B*). This was paralleled by the continued expression of c-myc (Fig. 1*C*), which was dramatically reduced in control preadipocytes

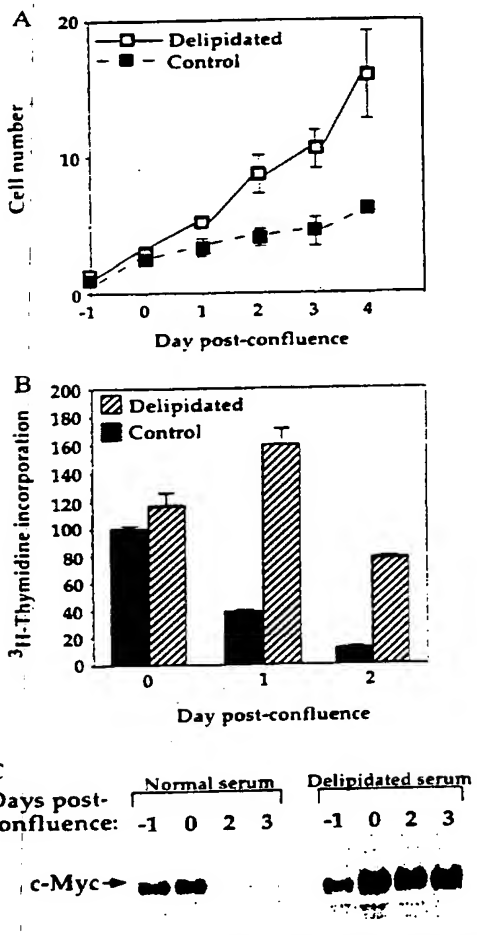

FIG. 1. Delipidated serum causes proliferation of preadipocytes beyond confluence. (*A*) Cell growth. Cells were switched to medium containing 10% delipidated serum or control just prior to confluence, and viable cell number was determined each day thereafter. Results are normalized to viable cell number at day 0. (*B*) Thymidine incorporation. Cells were incubated with [^{3}H]thymidine under each of the above conditions. Results are expressed as percentage of day 0 control. Shown is the mean ± SE of two experiments, with each data point done in triplicate in each experiment. (*C*) c-myc expression. Northern blot analysis for c-myc mRNA from cells cultured as in *A*. Loading was judged to be equal by ethidium bromide fluores-

shortly after confluence as described (8). The increase in cell density was apparent on inspection of the culture dishes (Fig. 2 *A–D*), and the cell shape was noted to be elongated as well (Fig. 2*B*). These morphological changes could be prevented by addition of a chloroform extract of the material removed from the delipidated serum (Fig. 2*E*).

Activators of PPAR Prevent and Reverse the Effects of Culturing Preadipocytes in Delipidated Serum. Since PPAR is activated by lipophilic agents, including fatty acids (13, 22, 23), we tested whether PPAR activators could substitute for the extracted substance(s) in this system. Remarkably, treatment with WY-14,643, a peroxisome proliferator and activator of PPAR, prevented and reversed the changes induced by the delipidated serum (Fig. 2 *F* and *G*). Continued exposure to WY-14,643 ultimately caused adipose conversion (Fig. 2*H*). Indeed, we found that clofibrate as well as the polyunsaturated fatty acid ETYA, another activator of PPAR, also caused adipose conversion of 3T3-L1 cells (Fig. 3 *C*, *E*, and *G*). The concentrations required to induce >90% of the cells to differentiate after 7 days were 3 mM clofibrate, 450 μM WY-14,643, and 50 μM ETYA. The relative potencies of these compounds paralleled those for activation of PPAR (13). Adipose conversion due to PPAR activators, like that

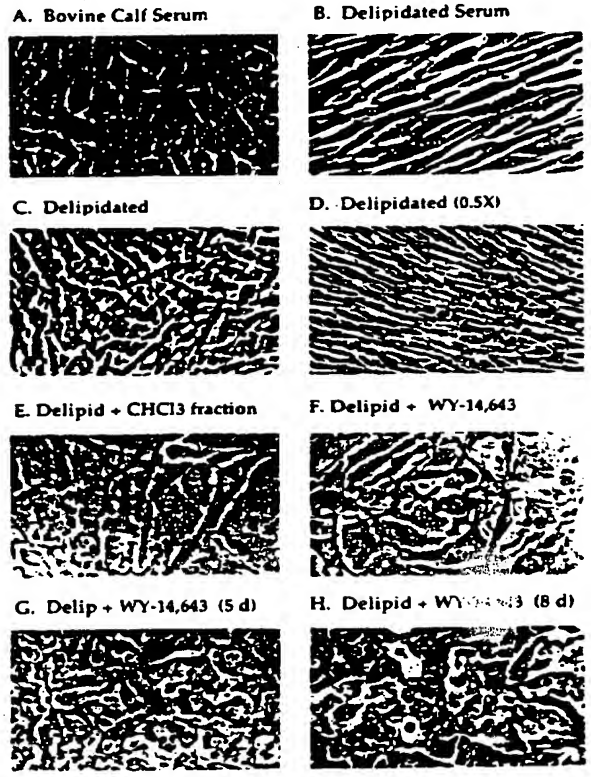

FIG. 2. Morphological effects of culturing preadipocytes in delipidated serum, and reversal by PPAR activators. 3T3-L1 preadipocytes were cultured under the conditions shown. Cells shown in *B* were less confluent at the time of change to medium containing delipidated serum (delipid) and resembled those shown in *C* after additional incubation time. Cells shown in *E* are representative of those near a drop of $CHCl_3$ extract of the heptane-soluble fraction of normal serum. $CHCl_3$ alone had no effect. The concentration of WY-14,643 used in *F–H* was 0.30 mM. Cells in *F* were exposed to delipidated serum and WY-14,643 simultaneously. Cells in *G* and *H* resembled those in *B* before addition of WY-14,643 for 5 and 8 days, respectively. Phase-contrast microscopy. (*A–C* and *E–H*, ×150; *D*, ×75.)

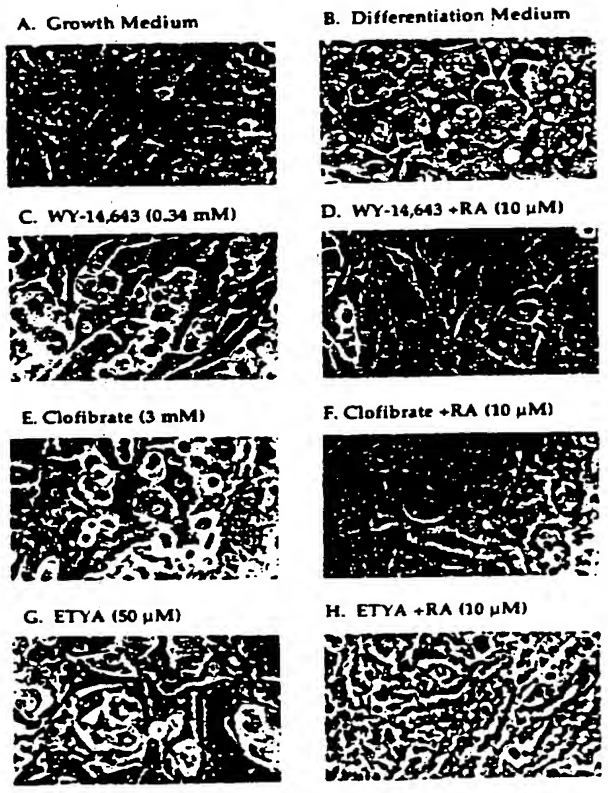

FIG. 3. Adipose conversion by PPAR activators. Cell morphology. Cells were cultured in conditions noted. Phase-contrast images are shown. (×150.)

due to standard differentiating conditions, was completely inhibited by RA (Fig. 3 *D*, *F*, and *H*).

Induction of PPAR and RXR During Adipose Conversion. The molecular events during adipose conversion by PPAR activators were compared with those of a standard differentiation protocol. WY-14,643, clofibrate, and ETYA (not shown) induced adipocyte-specific genes *aP2* and *C/EBPα* with a similar magnitude and time course as that due to standard conditions (Fig. 4*A*). In addition, PPARα expression increased ≈10-fold during adipose conversion by both standard differentiation conditions and WY-14,643 (Fig. 4*B*). The size of the PPAR mRNA was ≈7.0 kb, which is larger than initially reported (11) but the same as that found in other mouse tissues (unpublished observations) and in the rat (31). A second PPAR, Nuc-1 (29), was induced as well. The cells were also found to express the α, β, and γ subtypes of RXR (Fig. 4*C*). RXRα mRNA increased within 4 h after initiation of the standard differentiation protocol; this is the most rapid induction that has been demonstrated for any RXR and is one of the earliest molecular changes during adipose conversion. RXRγ was also induced, although considerably later than RXRα. The size of the RXRγ mRNA was ≈6 kb, which is larger than that previously reported (28) and raises the possibility that an unusual RXRγ isoform is expressed in fat.

RA Causes Apoptosis of Preadipocytes Cultured in Delipidated Medium. 3T3-L1 preadipocytes express RARα, -β, and -γ subtypes (unpublished data; see also ref. 32). However, while RA inhibited adipose conversion, it did not cause an appreciable phenotypic change in the cells cultured under usual conditions. Nevertheless, we were interested in the effects of RA on cells cultured in delipidated serum because [illegible] is depleted of retinoids. Remarkably, the cells

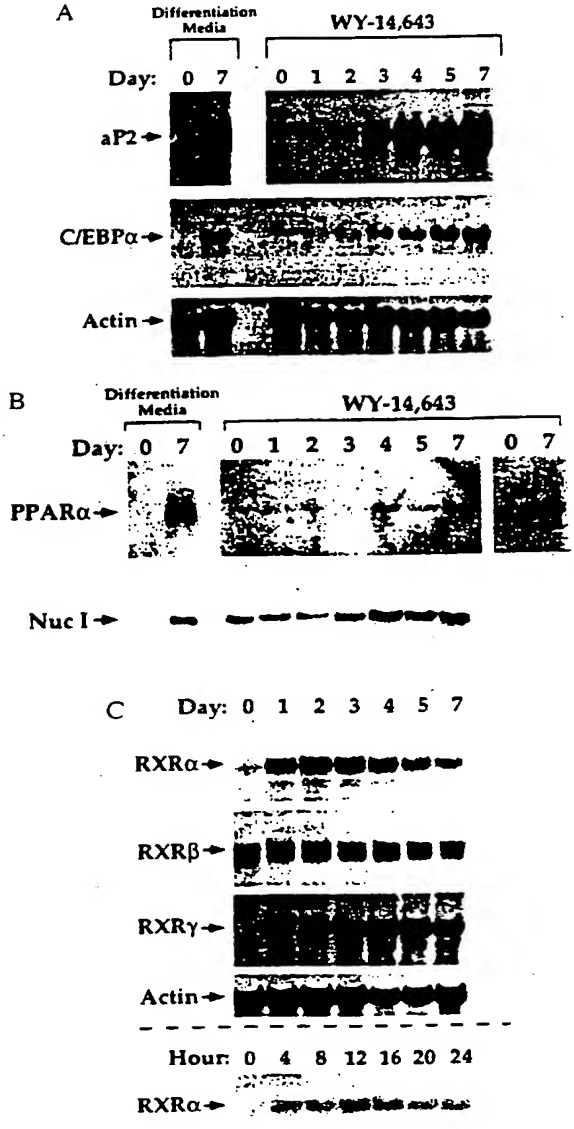

FIG. 4. Induction of PPAR and RXR during adipose conversion. (*A*) Comparison of molecular markers of adipose conversion induced by PPAR activators and standard conditions. Northern blot analysis of aP2, C/EBPα, and actin mRNA during differentiation by IBMX, Dex, FCS, and insulin (differentiation media) and WY-14,643 (0.45 mM). (*B*) Induction of PPAR and Nuc-1. Differentiation by WY-14,643 (0.45 mM) and by differentiation medium. Fifteen micrograms of total RNA was used in WY-14,643 experiments, while 4 μg of poly(A)$^+$ RNA from cells differentiated by standard conditions was used. (*C*) Induction of RXR. Fifteen micrograms of total RNA per lane. Loading was judged equal by actin hybridization as well as ethidium bromide fluorescence of rRNA.

developed cytoplasmic blebbing, vacuolization, and condensation within 3–4 days of exposure to RA (Fig. 5 *C* and *D*), changes characteristic of cells undergoing apoptosis (33). Quantitatively, viable cell number was decreased by >90% 5 days after exposure to RA (Fig. 5*E*). Fig. 5*F* shows that chromosomal DNA from the dying cells displayed the periodic (≈200 bp) fragmentation pattern frequently seen in

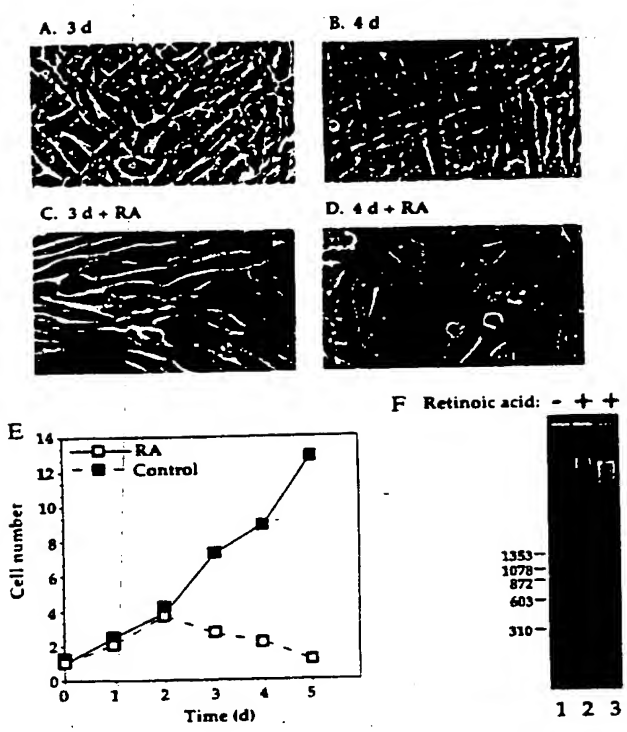

FIG. 5. RA induces apoptosis of preadipocytes cultured in delipidated serum. (*A–D*) Morphological changes in RA-treated cells. Cells were cultured for 3 days (*A* and *C*) and 4 days (*B* and *D*) in delipidated serum-containing media. In *C* and *D*, RA was present for the first 48 h. (*E*) Cell death due to RA. Cell number after incubation in delipidated serum-containing medium in the presence or absence of RA, normalized to cell number at day 0. (*F*) Chromosomal DNA fragmentation induced by RA. Cells were cultured in delipidated serum-containing medium for up to 5 days, with 10 μM RA added for the first 48 h (lanes 2 and 3). Lanes: 1, 5 days, no RA; 2, 4 days, plus RA; 3, 5 days, plus RA. Little genomic DNA is seen in lane 1 because the method used selected for fragmented DNA, which was nearly absent in these growing cells. Migration of DNA size markers is shown on the right. Numbers on left are bp.

apoptosis (compare lanes 1 and 3). The ED_{50} for induction of apoptosis by RA was ≈0.5 μM. 9-*cis*-RA, a ligand for RAR as well as RXR, had similar effects (data not shown).

DISCUSSION

The effects of incubation in delipidated serum reveal that usual culture conditions constrain the 3T3-L1 preadipocyte in a state that is intermediate between proliferative and terminally differentiated cells. Since this is prevented by activators of PPAR, we speculate that the delipidation process might have removed an endogenous activator of PPAR, perhaps the PPAR ligand itself. The correlation between the potency of PPAR activators and their abilities to induce adipose conversion further suggests that PPAR is mechanistically involved. Nevertheless, PPAR is also expressed in a variety of nonadipose tissues where it undoubtedly mediates other effects.

The simultaneous induction of PPAR and the accumulation of lipid within the adipocyte might serve to maintain the adipocyte phenotype, since PPAR is activated by fatty acids (13, 22, 23). Indeed, adipose-specific transcription of the *aP2* gene is stimulated by fatty acids (34). During adipose con-

in the heterodimer form. The main RXR partner is likely to vary during the course of differentiation since RXRα is rapidly induced, while RXRγ is induced to an even greater extent at later times. Understanding the role of endogenous activators of PPAR, RXR, and RAR in regulating adipocyte differentiation may allow the rational design of agents to prevent and treat obesity.

The preadipocyte cultured in delipidated serum displays a remarkable susceptibility to RA-induced cell death by apoptosis. It is known that RA sets in motion complex differentiation programs involving programmed cell death, such as limb development (35), and some HL-60 leukemic cells die via apoptosis after being induced to differentiate by RA (36). The present report provides an example of RA-induced apoptosis of a clonal, dividing cell population. It is of interest that 9-*cis*-RA also causes apoptosis of the preadipocytes, whereas it protects T cells from a similar fate (37). Relatively high concentrations of RA are required for induction of apoptosis in preadipocytes, suggesting that the RA may be acting via conversion to other retinoids. Nevertheless, the concentrations of RA that caused apoptosis were not toxic to adipocytes or to preadipocytes cultured in normal serum. It is possible that RA induces gene products that prevent differentiation in the presence of PPAR activators but are deleterious to cells cultured in delipidated serum. In any case, the diverse effects of RA on cells cultured in the absence and presence of PPAR activators indicate that convergence of these two nuclear receptor-mediated pathways regulates the fate of the preadipocyte.

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Fibrates Increase Human REV-ERBα Expression in Liver via a Novel Peroxisome Proliferator-Activated Receptor Response Element

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Fibrates are widely used hypolipidemic drugs that act by modulating the expression of genes involved in lipid and lipoprotein metabolism. Whereas the activation of gene transcription by fibrates occurs via the nuclear receptor peroxisome proliferator-activated receptor-α (PPARα) interacting with response elements consisting of a direct repeat of the AGGTCA motif spaced by one nucleotide (DR1), the mechanisms of negative gene regulation by fibrates and PPARα are largely unknown. In the present study, we demonstrate that fibrates induce the expression of the nuclear receptor Rev-erbα, a negative regulator of gene transcription. Fibrates increase Rev-erbα mRNA levels both in primary human hepatocytes and in HepG2 hepatoblastoma cells. In HepG2 cells, fibrates furthermore induce Rev-erbα protein synthesis rates. Transfection studies with reporter constructs driven by the human Rev-erbα promoter revealed that fibrates induce Rev-erbα expression at the transcriptional level via PPARα. Site-directed mutagenesis experiments identified a PPAR response element that coincides with the previously identified Rev-erbα negative autoregulatory Rev-DR2 element. Electromobility shift assay experiments indicated that PPARα binds as heterodimer with 9-*cis*-retinoic acid receptor to a subset of DR2 elements 5′ flanked by an A/T-rich sequence such as in the Rev-DR2. PPARα and Rev-erbα bind with similar affinities to the Rev-DR2 site. In conclusion, these data demonstrate human Rev-erbα as a PPARα target gene and identify a subset of DR2 sites as novel PPARα response elements. Finally, the PPARα and Rev-erbα signaling pathways cross-talk through competition for binding to those response elements. (Molecular Endocrinology 13: 400–409, 1999)

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INTRODUCTION

Fibrates are hypolipidemic drugs that lower plasma cholesterol and triglycerides (1). Fibrates exert their effects primarily via the liver by regulating the expression of several genes implicated in lipid metabolism. On the one hand, fibrates stimulate the expression of the human apo A-I (2), rat lipoprotein lipase (3), rat acyl-CoA synthetase (4), rat acyl-CoA oxidase (5), rat multifunctional enzyme (6), and human muscle-type

carnitine palmitoyltransferase I (7) genes in the liver. On the other hand, fibrates repress the expression of the rat apo A-I (8), rat apo A-IV (9), human, rat, and mouse apo C-III (10–13), rat hepatic lipase (14), and rat lecithin-cholesterol acyl transferase (15) genes in the liver. Fibrates have been shown to activate specific receptors, termed peroxisome proliferator-activated receptors (PPARs), belonging to the nuclear receptor gene superfamily (16–18). So far, three different PPAR forms, α, β(δ), and γ, have been identified, of which the PPARα form mediates the effects of fibrates on liver gene expression (13, 19). After activation, PPARs heterodimerize with the 9-*cis*-retinoic acid receptor (RXR) and subsequently bind to DNA on specific response elements termed peroxisome proliferator response elements (PPRE), located in regulatory regions of target genes, thereby modulating their transcriptional activity. All PPREs identified so far consist of the juxtaposition of two derivatives of the canonical hexamer sequence PuGGTCA spaced by one nucleotide and commonly called direct repeat 1 (DR1).

Whereas PPARα mediates fibrate action on lipoprotein metabolism through PPREs identified in the regulatory sequences of positively regulated genes, the mechanisms of negative gene regulation by fibrates are unclear. Studies using PPARα knockout mice demonstrated that PPARα is a mediator of the negative regulation by fibrates, at least with respect to the mouse apo A-I and apo C-III genes (13). Fibrates may repress transcription by interfering negatively with the expression and activity of positive transcription factors, such as hepatocyte nuclear factor-4 (HNF-4) (11, 20). However, not all fibrate-regulated genes are under transcriptional control by HNF-4. For instance, although fibrates repress rat apo A-I gene transcription, HNF-4 is not considered to be a major regulator of apo A-I gene transcription (21, 22). Alternatively, fibrates may actively repress transcription by activating a negative transcription factor. Interestingly, we recently identified in the rat apo A-I gene promoter a response element for the nuclear receptor Rev-erbα, an orphan receptor of the nuclear receptor family that acts as a negative transcription factor (23). Furthermore, we have shown that Rev-erbα gene expression is induced by fibrates in rat liver, indicating that Rev-erbα may be a mediator of negative gene transcription by fibrates.

The goal of the present study was to determine whether fibrates also regulate human Rev-erbα expression and to investigate the molecular mechanisms involved. Our results demonstrate that fibrates increase Rev-erbα expression in human hepatocytes and in HepG2 cells. Furthermore, we show that the induction of Rev-erbα gene expression occurs at the transcriptional level in hepatocytes and is mediated by PPARα. Finally, we demonstrate that PPARα binds to a DR2 site coinciding with the Rev-DR2 site in the human Rev-erbα promoter (24), which constitutes a novel PPARα response element mediating a cross-talk between the PPARα and Rev-erbα pathways.

RESULTS

Fibrates Increase Rev-erbα mRNA Expression and Protein Synthesis in Human Liver Cells

The regulation of Rev-erbα by fibrates was analyzed in human primary hepatocytes. Treatment of cells with fenofibric acid or Wy 14,643 induced a pronounced increase of Rev-erbα mRNA levels, whereas control 36B4 mRNA levels did not change (Fig. 1A). In addition, in HepG2 cells treatment with Wy 14,643 increased Rev-erbα mRNA levels in a dose-dependent fashion (Fig. 1B). To analyze whether the induction of Rev-erbα mRNA by fibrates is associated with increased synthesis of Rev-erbα protein, HepG2 cells were cultured for 24 h in the presence of Wy 14,643 or vehicle, labeled with ^{35}S-methionine, and Rev-erbα was subsequently immunoprecipitated. Compared with control, treatment with Wy 14,643 resulted in a significant increase in Rev-erbα protein synthesis (Fig. 1C). By contrast, as a control, apolipoprotein E secretion was not influenced by fibrate treatment (data not shown). These experiments demonstrate that fibrates increase Rev-erbα mRNA levels as well as protein synthesis in human hepatocytes.

Fibrate Induction of Rev-erbα Gene Expression Occurs at the Transcriptional Level via PPARα Interacting with the Rev-DR2 Site of the Human Rev-erbα Promoter

To investigate whether the effect of fibrates on Rev-erbα expression occurred at the transcriptional level, the 1.7 kb containing Rev-erbα promoter was transiently transfected in HepG2 cells in the presence of a human PPARα expression vector (pSG5hPPARα) or empty vector (pSG5) (Fig. 2A). Rev-erbα promoter-driven luciferase activity increased significantly after cotransfection with PPARα, an effect that was increased in the presence of fenofibric acid (Fig. 2A), indicating that Rev-erbα gene transcription is increased by PPARα. Two putative nuclear receptor-binding sites containing AGGTCA-like motifs were previously identified in the human Rev-erbα promoter and called distal (Rd) and proximal sites (Rp) (24). To delineate whether one of these putative binding sites mediated PPARα transactivation, unilateral deletion and site- directed mutagenesis experiments were performed. Hence, a 0.7-kb 5′-deletion of the Rev-erbα promoter containing only the Rp site was transfected in the presence or absence of human PPARα (Fig. 2A). This deleted Rev-erbα promoter construct was induced by PPARα. Since both the 1.7-kb and 0.7-kb Rev-erbα promoter constructs responded to the same extent to PPARα, we hypothesized the existence of a PPRE located near the Rp site of the human Rev-erbα promoter (24). Thus, to determine the role of this site in the transcriptional regulation of Rev-erbα

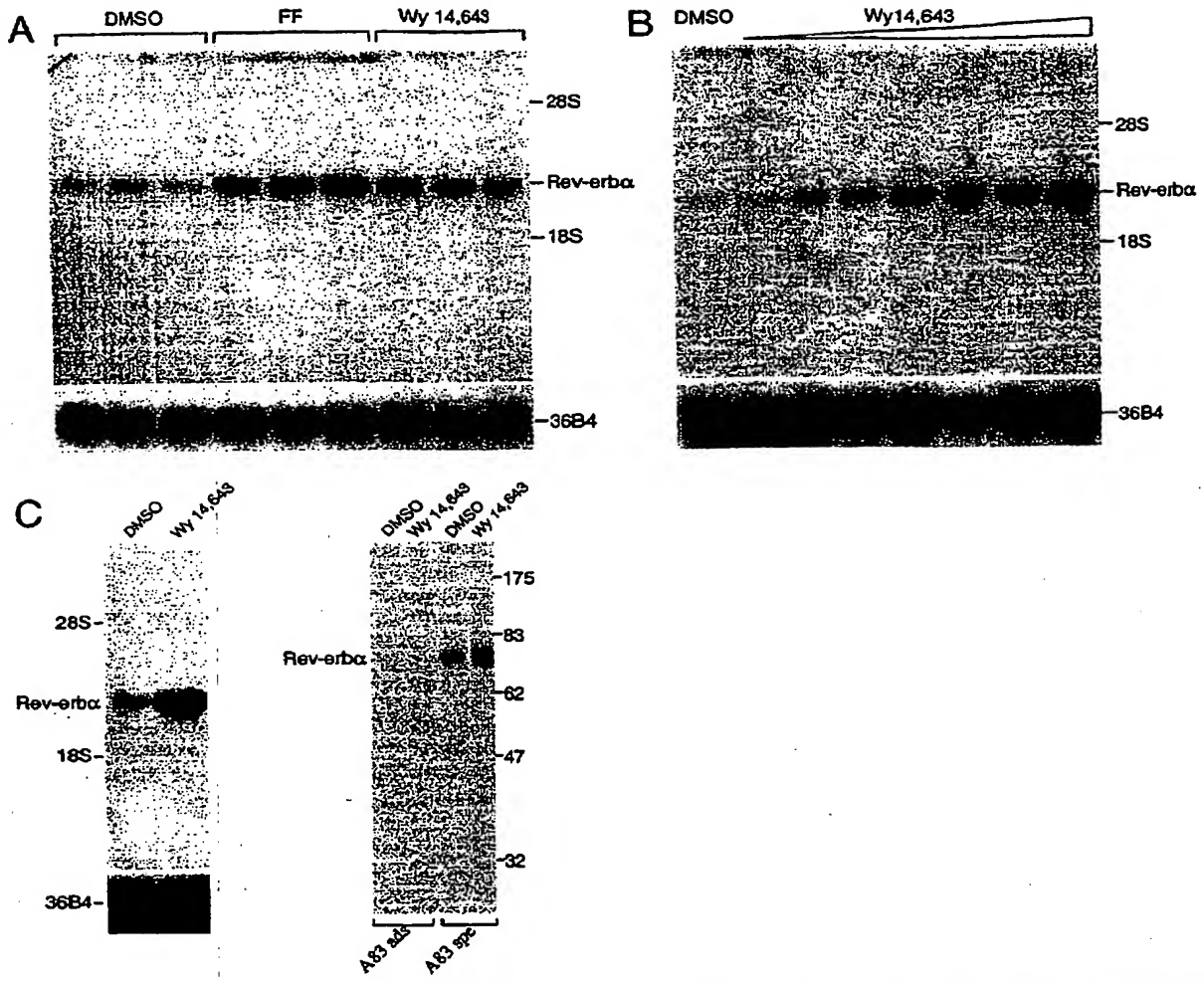

Fig. 1. Fibrates Increase Rev-erbα mRNA Expression and Protein Synthesis in Primary Human Hepatocytes and HepG2 Cells

Total RNA (10 μg) was subjected to Northern blot analysis using hRev-erbα (*top panel*) or 36B4 (*bottom panel*) cDNA probes as described in *Materials and Methods*. A, Human hepatocytes were isolated and treated for 24 h with 100 μM fenofibric acid, 50 μM Wy 14,643, or vehicle (DMSO). B, HepG2 cells were treated for 24 h with increasing concentrations of Wy 14,643 (0, 10, 50, 100, 150, 200, 250, 500 μM). C, HepG2 cells were treated for 24 h with 500 μM Wy 14,643 or DMSO. *Left*, RNA analysis from the same plates used for immunoprecipitation experiments. *Right*, Cell lysates were subjected to immunoprecipitation using serum depleted of anti-Rev-erbα antibody (A83 ads) or polyclonal anti-Rev-erbα antibody (A83 spe) as described in *Materials and Methods*.

by PPARα, we explored the influence of PPARα on various mutations around this region (Fig. 2A). Mutations affecting either the 5′-AGGTCA motif (pGL2hRev-erbα Δ) or the A/T-rich region (pGL2hRev-erbα CCC) of the Rp site resulted not only in a loss of Rev-erbα promoter inductibility by PPARα, but also in an increase in baseline reporter activity (Fig. 2A). These results indicate that the PPARα response element colocalizes with the proximal Rev-erbα binding site, referred to as Rev-DR2 (24).

To ascertain that the Rev-DR2 site could function as a PPAR-responsive element, we performed transient transfection experiments using wild-type and mutated versions of the Rev-DR2 site cloned in front of the heterologous SV40 promoter (Rev-DR2 SV40, M5′Rev-DR2 SV40, and M3′Rev-DR2 SV40) (Fig. 2B). Upon cotransfection with pSG5hPPARα in HepG2 cells, it was evident that the Rev-DR2 could transmit PPARα responsiveness to the heterologous SV40 promoter, an effect that was enhanced in the presence of fenofibric acid. By contrast, PPARα did not activate the SV40 promoter. Furthermore, PPARα did not induce the activity of the SV40 promoter driven by the Rev-DR2 site mutated in its 5′-half-site (Fig. 2B), confirming the importance of

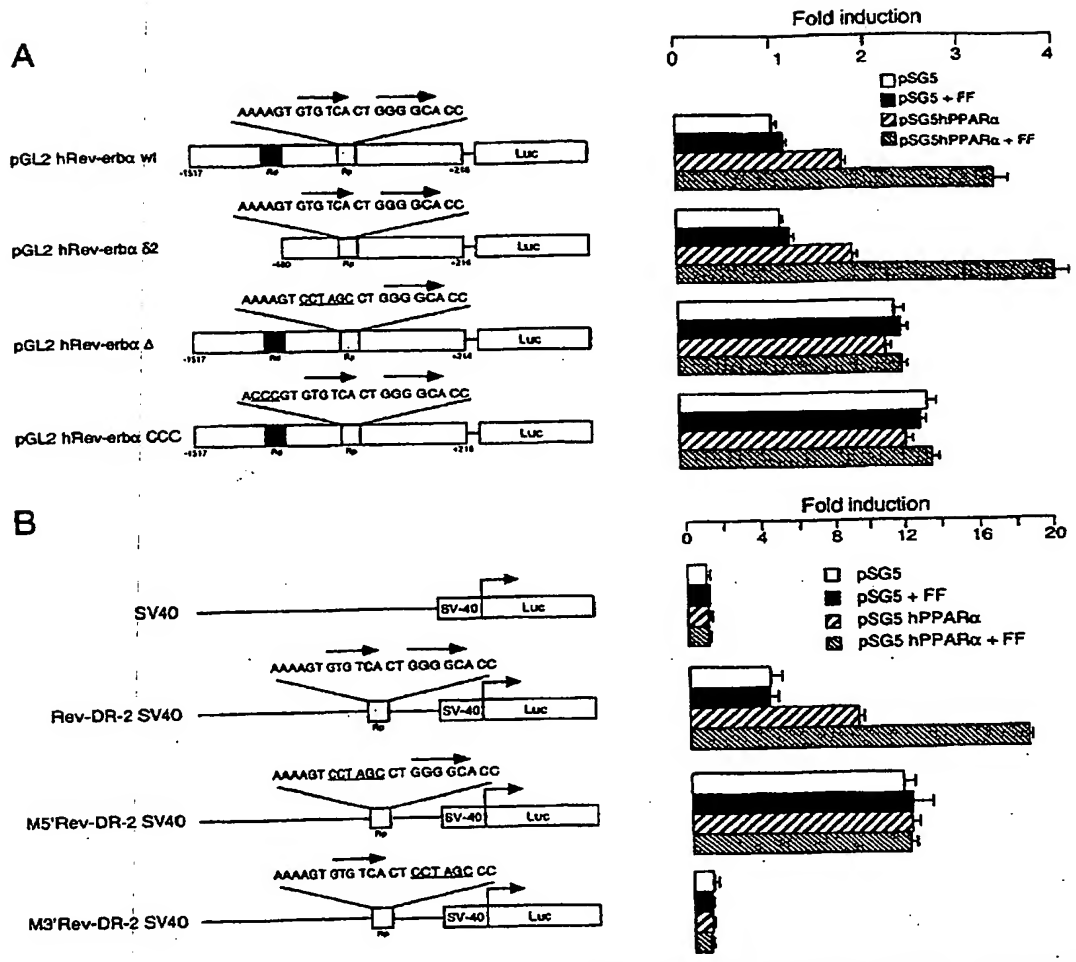

Fig. 2. Fibrate Induction of Rev-erbα Gene Expression Occurs at the Transcriptional Level via PPARα Interacting with the Rev-DR2 Site of the Human Rev-erbα Promoter

A, Effects of PPARα on the expression of human Rev-erbα promoter containing a wild-type or mutated Rev-DR2. B, Effects of PPARα on wild-type or mutated human Rev-erbα Rev-DR2 cloned in two copies upstream of the heterologous SV40 promoter. Luc, luciferase reporter gene. HepG2 cells were transfected with the indicated reporter constructs, in the presence of cotransfected pSG5hPPARα or pSG5 vector. Cells were treated with fenofibric acid (FF) (10 μM) or vehicle (DMSO), and luciferase activity was measured as described in *Materials and Methods*. The Rev-DR2 half-site direct repeat sequences are indicated by *arrows*. Rd and Rp putative nuclear receptor-binding sites are shown as *solid and shaded boxes*, respectively. The mutated nucleotides in the Rp site are *underlined*.

this motif in the structure of the PPARα-responsive element. Interestingly, mutation of the 3′-half-site of the DR2 also abolished transactivation by PPARα (Fig. 2B), indicating that the 3′-AGGTCA half-site is also implicated in mediating induction of human Rev-erbα gene transcription by PPARα. Taken together, these data strongly argue that the human Rev-erbα promoter contains a bona fide PPAR-responsive element that coincides with the Rev-DR2 site, which is constituted of two AGGTCA motifs separated by two nucleotides (DR2) and 5′-flanked by an A/T-rich region (Rev-DR2).

PPAR Binds as a Heterodimer with RXR to a DR2 Site Containing an A/T-Rich 5′-Flanking Region, but Not to a Standard DR2 Site

To investigate direct interaction of PPARα with the Rev-DR2 site, we performed electromobility shift assays (EMSAs) using *in vitro* synthesized PPARα and RXRα protein. RXRα or PPARα alone did not bind to the Rev-DR2 site oligonucleotides (Fig. 3B, lanes 10–12 and 14 and lanes 15–17 and 19). Furthermore, PPARα did not bind to an oligonucleotide containing a monomer binding site for Rev-erbα (G8A) (Fig. 3B, lane

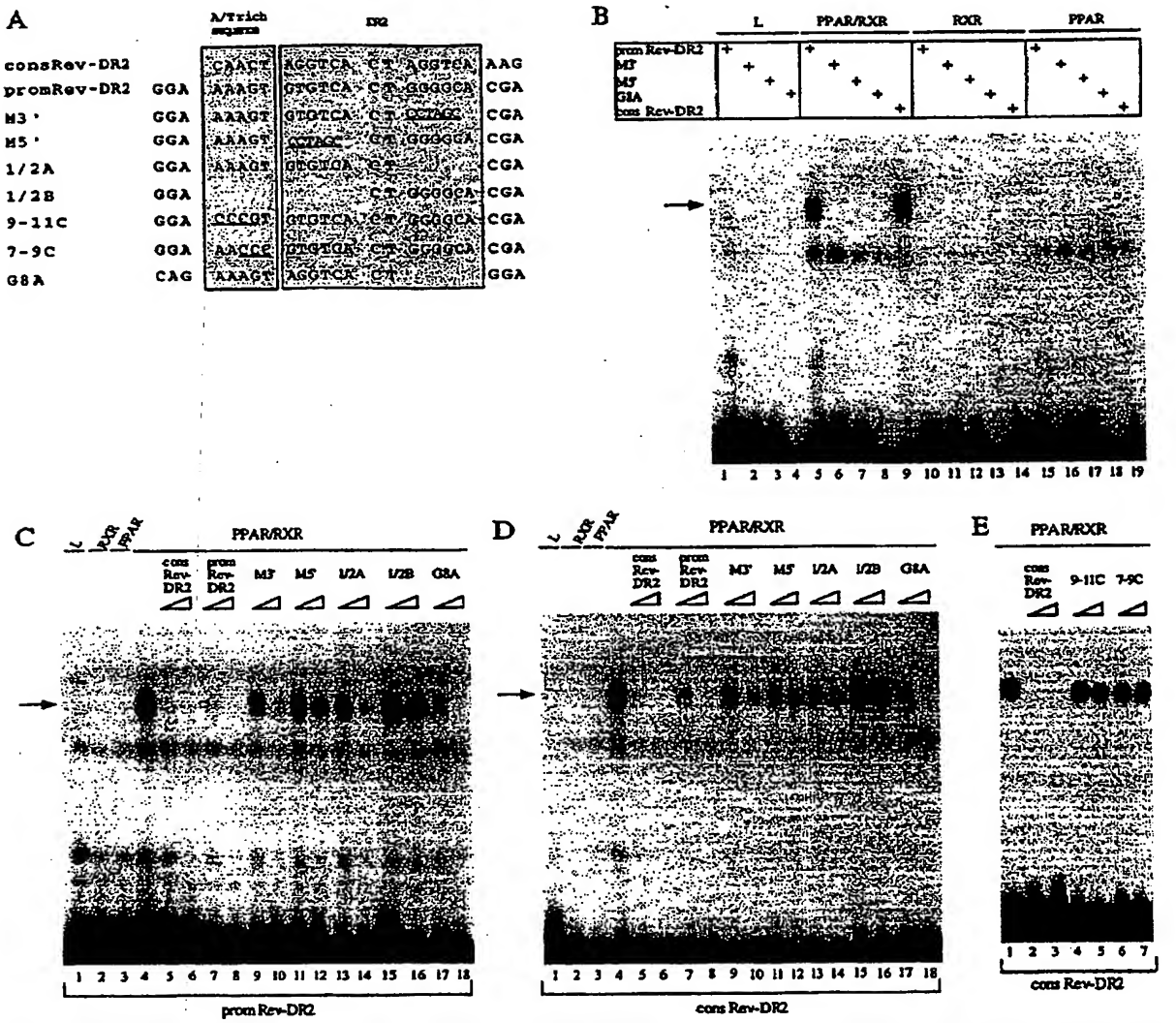

Fig. 3. PPARα Binds as a Heterodimer with RXRα to a DR2 Site Containing an A/T-Rich 5′-Flanking Region, but Not to a Standard DR2 Site

A, Sequences of the different response elements used as probes or as competitors. The 5′-flanking A/T-rich region and half-site sequences are indicated. The mutated sequences are *underlined*. B, Gel retardation assays were performed on the indicated end-labeled oligonucleotides in the presence of *in vitro* translated hPPARα and mRXRα or unprogrammed lysate (Unprog. L) (B–E). Competition experiments for binding of PPARα/RXRα to the human Rev-erbα promoter Rev-DR2 (prom Rev-DR2) (C) or consensus Rev-DR2 (cons Rev-DR2) (D) and (E) oligonucleotides were performed with 10- and 100-fold molar excess of indicated cold oligonucleotide. PPAR/RXR heterodimer or Rev-erbα complexes are indicated by *arrows*.

18) (24), indicating that PPARα cannot bind as a monomer. By contrast, binding was observed when PPARα was incubated in the presence of RXR with the Rev-erbα promoter Rev-DR2 as well as the consensus Rev-DR2 sites (direct repetition of the AGGTCA motif separated by two nucleotides), which both contain an A/T-rich region at their 5′-extremities (Fig. 3B, lanes 5 and 9). This binding was specific since it was competed out by excess of unlabeled oligonucleotide (Fig. 3, panel C, lanes 4–8, and panel D, lanes 4–8).

Next, we characterized the structural requirements for PPAR/RXR binding by performing competition EMSAs on the promoter (Fig. 3C) as well as on the consensus (Fig. 3D) oligonucleotides. PPAR/RXR binding to wild-type promoter Rev-DR2 (Fig. 3C, lane 4) or to consensus Rev-DR2 (Fig. 3D, lane 4) oligonucleotides could not be competed either by the promoter Rev-DR2 site carrying a mutation in the 5′-half-site (M5′) (24) or in the 3′-half-site (M3′) (Fig. 3, C and D, lanes 9, 11, and 12). Interestingly, at high excess (100-fold) of

competitor, the 3′-half-site-mutated oligonucleotide started competing for PPAR/RXR binding, indicating that the 3′-part of the Rev-DR2 site is of lesser importance (Fig. 3, C and D, lane 10). Similar results were obtained with an oligonucleotide containing the promoter Rev-DR2 site completely lacking the second half-site (1/2A) (Fig. 3, C and D, lanes 13 and 14), whereas an oligonucleotide lacking the promoter Rev-DR2 A/T-rich sequence and 5′-half-site (1/2B) did not compete at all (Fig. 3, C and D, lanes 15 and 16). These results indicate that 5′- and 3′-half-sites are both implicated in PPAR/RXR binding, with the 5′-half-site, to which PPARα presumably binds (25), being most important.

To investigate the role of the 5′-A/T-rich flanking sequence in PPAR/RXR binding to a DR2 site, competition experiments were performed with oligonucleotides in which the 5′-flanking sequence was substituted by C nucleotides (9–11C and 7–9C; Fig. 3A). Neither 9–11C nor 7–9C oligonucleotides competed for PPAR/RXR binding to the consensus Rev-DR2 sequence (Fig. 3E), indicating absolute requirement of the 5′-A/T-rich flanking sequence for PPAR/RXR binding to a DR2 site.

To ensure that Rev-DR2 sites are high-affinity response elements for PPARα, we compared the relative affinities of PPAR/RXR binding to either DR1 or Rev-DR2 sites by competition EMSA (Fig. 4). Using oligonucleotides labeled to similar specific activities and under identical experimental conditions, a higher intensity shift with PPAR/RXR was obtained on the Rev-DR2 oligonucleotide compared with the naturally occurring DR1 PPRE site of the human apo A-II promoter, which has been shown to drive its regulation by fibrates (26) (Fig. 4, lanes 1 and 10). When increasing amounts of unlabeled oligonucleotide were added, cold Rev-DR2 oligonucleotide competed more efficiently than cold DR1 oligonucleotide for binding of PPAR/RXR to the Rev-DR2 site (Fig. 4, lanes 2–5 and 6–9). Reciprocally, binding of PPAR/RXR to labeled DR1 oligonucleotide was more rapidly competed by cold Rev-DR2 than by cold DR1 oligonucleotide (Fig. 4, lanes 11–14 and 15–18).

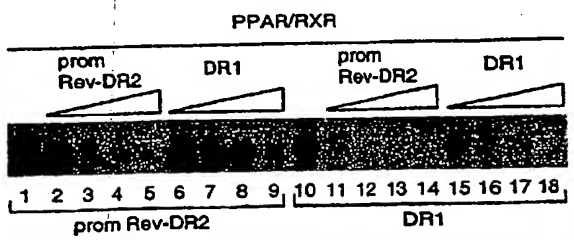

Fig. 4. PPARα Binds with Similar Affinities to Natural DR2 and DR1 PPAR Response Element

Gel retardation assays were performed on end-labeled RevDR2 (prom Rev-DR2) and apo A-II PPRE DR1 oligonucleotides (29) in the presence of *in vitro* transcribed/translated PPARα and RXRα protein. Competition experiments were performed by adding 1-, 5-, 10-, and 50-fold molar excess of indicated oligonucleotide.

These binding experiments demonstrate that PPARα binds as a heterodimer with RXR, but not as monomer, to DR2 sites containing a Rev-erbα-type 5′-flanking region and that Rev-DR2 constitutes a novel PPARα-binding site of higher affinity than the natural apo A-II DR1 PPRE site.

Rev-DR2 Mediates a Cross-Talk between PPARα and Rev-erbα

To test directly whether PPARα and Rev-erbα could functionally compete on a Rev-DR2 element, transient cotransfection experiments were performed. As expected, Rev-erbα was able to repress Rev-DR2-driven SV40 promoter activity (24) (Fig. 5A). Cotransfection of PPARα in increasing proportions against a constant amount of Rev-erbα led to a progressive abolishment of Rev-erbα-mediated repression resulting in a transcriptional activation of the reporter gene at a 3:2 ratio of PPARα to Rev-erbα, respectively, as evidenced by Western blot analysis of transfected cell extracts (Fig. 5A and *inset*). Furthermore, in the absence of cotranfected Rev-erbα, reporter transcription activity was even further enhanced by PPARα (Fig. 5A). Thus, PPARα and Rev-erbα are able to functionally cross-compete for the same Rev-DR2 element.

Finally, to estimate the relative affinities of PPAR/RXR and Rev-erbα binding to a Rev-DR2 site, EMSAs were performed using Rev-DR2 as probe (Fig. 5B). As expected, PPAR/RXR formed a heterodimeric complex whereas Rev-erbα bound both as monomer and as heterodimer (Fig. 5B, lanes 1 and 6). When competition was performed using cold Rev-DR2 oligonucleotide, PPAR/RXR binding decreased in a manner similar to Rev-erbα monomer (Fig. 5B, compare lanes 2–5 and 7–10). However, Rev-erbα homodimer binding appeared slightly more sensitive to competition with unlabeled Rev-DR2 (Fig. 5B, lanes 7–10). Altogether, these results indicate that PPAR/RXR binds to Rev-DR2 sites with similar affinity as Rev-erbα monomer, whereas Rev-erbα homodimers appear to bind with higher affinity.

DISCUSSION

In the present report we studied the regulation of Rev-erbα by fibrates in human liver cells and the molecular mechanisms involved. Our results on human primary hepatocytes and HepG2 cells demonstrate that fibrates induce Rev-erbα mRNA expression, an effect that is associated with induction of Rev-erbα protein synthesis in HepG2 cells. In addition, transfection studies revealed that the regulation of Rev-erbα expression by fibrates occurs at the transcriptional level via PPARα. Using deleted and mutated Rev-erbα promoter constructs, we localized the fibrate-respon-

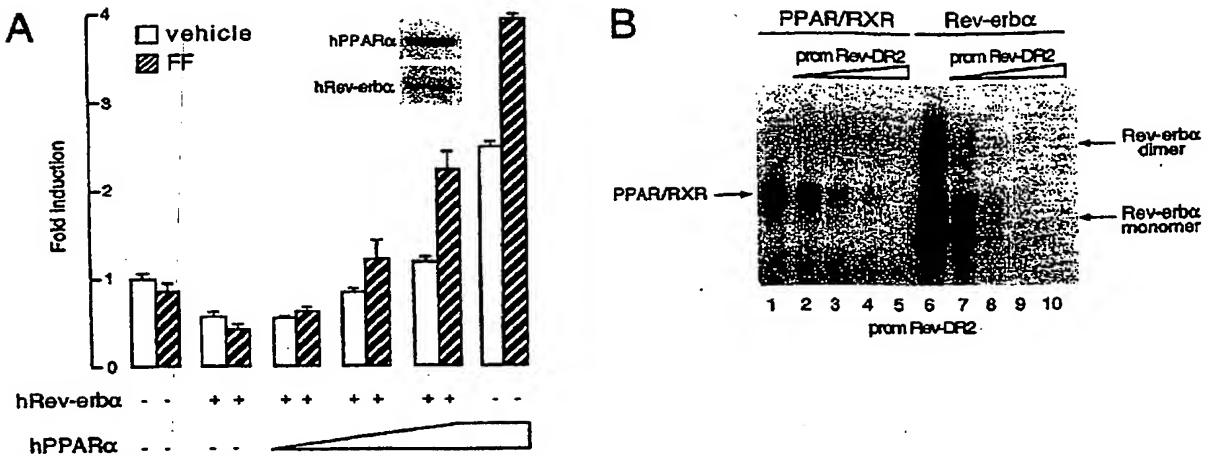

Fig. 5. Rev-DR2 Mediates a Cross-Talk between PPARα and Rev-erbα

A, PPARα relieves the repressive activity of Rev-erbα on Rev-DR2-driven transcription. HepG2 cells were transfected with the Rev-DR2 SV40pGL2 reporter construct (Fig. 2B), in the presence of pSG5hPPARα and/or pSG5hRev-erbα expression vector. Increasing amounts of pSG5hPPARα (0.5-fold, 1-fold, 1.5-fold) were added to a constant amount of pSG5hRev-erbα. Cells were treated with fenofibric acid (FF) (10 μM) or vehicle (DMSO), and luciferase activities were measured as described in *Materials and Methods* and expressed relative to control set as 1. Proteins extracted from HepG2 cells transfected with Rev-erbα and 1.5-fold excess of PPARα were analyzed by immunoblotting (*inset*). B, Competition experiments for binding of PPARα/RXRα or Reverbα to the human Rev-erbα promoter Rev-DR2 (prom Rev-DR2) oligonucleotide. Competition was performed with 1-, 5-, 10-, and 50-fold molar excess of indicated cold oligonucleotide. PPAR/RXR heterodimer and Rev-erbα monomer or homodimer complexes are indicated by *arrows*.

sive region in the human Reverbα promoter to the previously identified negative Rev-erbα autoregulation site. Moreover, mutations in the Rev-DR2 site of the Rev-erbα promoter abolished basal Rev-erbα-mediated repression as well as PPARα-mediated activation. EMSA experiments proved that fibrate signaling occurs through direct interaction of PPAR/RXR heterodimers to the Rev-DR2 site of the human Rev-erbα promoter. Since all PPREs described so far consist of the juxtaposition of the degenerated hexamer AGGTCA sequence separated by one nucleotide (DR1) (4, 6, 11, 26–38), these results represent the first demonstration of a DR2 site as a PPAR-responsive element. Interestingly, a specific structure of the DR2 is required for high-affinity PPAR/RXR binding. In addition to the 5′- and 3′-AGGTCA half-sites, the 5′-flanking region is required for binding of PPAR/RXR to a DR2 site. Thus, several fundamental characteristics of protein-DNA interaction, such as the contact of the receptor with the 5′-A/T-rich flanking sequences of the response element, are conserved among a number of the superfamily members. Taken together, our data suggest that nuclear receptors are more flexible for recognition of responsive elements than previously anticipated.

Rev-erbα belongs to a subfamily of orphan receptors that are repressors of target gene transcription (for review see Ref. 39). Rev-erbα appears to be ubiquitously expressed (40, 41), but its functions are ill defined. Several observations suggest a role for Rev-erbα in metabolic control and energy homeostasis. First, Rev-erbα mRNA levels increase during differentiation of preadipocytes into adipocytes (42). Second, Rev-erbα has been suggested to act as a modulator of thyroid hormone signaling (40, 41, 43, 44). Indeed, Rev-erbα has been shown to bind a subset of thyroid hormone-response elements (45). Interestingly, a significant level of cross-talk exists also between peroxisome proliferator and thyroid hormone-signaling pathways (46–52). Our present data identify Rev-erbα as a fibrate target gene and reveal the existence of cross-talk between the PPARα and Rev-erbα-signaling pathways. This cross-talk is governed via two mechanisms (see Fig. 6 for overview). First, PPARα induces Rev-erbα expression by interfering with the negative autoregulatory loop of Rev-erbα expression via the Rev-DR2 site. Therefore, genes regulated by Rev-erbα, such as N-*myc* (53) and rat apo A-I (23), will be negatively regulated by PPARα via an indirect mechanism. Second, PPARα and Rev-erbα may compete for binding to similar DR2 sites. Rev-erbα itself is an example of a gene containing a response element recognized by both PPARα and Rev-erbα. Hence, target genes containing Rev-DR2 sequences to which PPARα, as heterodimer with RXR, and Rev-erbα compete for binding will be derepressed by fibrates. By contrast, genes carrying monomeric Rev-RE, to which Rev-erbα binds exclusively as monomer, will be further repressed after fibrate treatment. Therefore, whether a gene will be predominantly regulated by PPARα or Rev-erbα will depend on the relative levels of ligands for each receptor, the relative concentra-

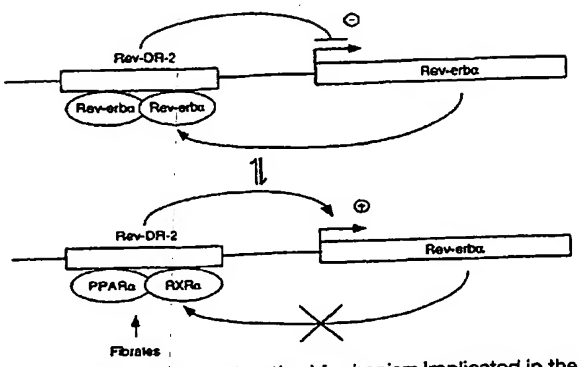

Fig. 6. Scheme Describing the Mechanism Implicated in the Regulation of Rev-erbα Expression by Fibrates

It is conceivable that an equilibrium exists between inducible activation of the Rev-erbα promoter by PPAR/RXR heterodimer and its repression by Rev-erbα. This allows a fine tuning of Rev-erbα mRNA and protein levels and hence of Rev-erbα target gene expression.

tions of each receptor, and on the structure of the target gene DR2 sequence that determines the relative binding affinities of PPARα and Rev-erbα. The fact that PPARα and Rev-erbα bind to similar DR2 subset sites is most likely due to the similarity of their T/A boxes (25), which are involved in the recognition of the 5′-half-site extension (24, 45, 54–56). Therefore, PPARα could repress genes by inducing Rev-erbα while simultaneously activating its own target genes via either DR1 or DR2 sites.

In a previous study, we demonstrated that differences between human and rat apo A-I gene regulation in response to fibrates are due to a combination of two distinct mechanisms implicating the nuclear receptors PPARα and Rev-erbα (23). Our data indicated that the species-distinct regulation of apo A-I gene expression by fibrates is due to sequence differences in *cis*-acting elements. In man, apo A-I transcription is induced via PPARα binding to a positive PPRE located in the A site footprint (2). This site is not conserved in rats, resulting in a lack of binding of PPAR to the rat apo A-I A promoter site. By contrast, rat apo A-I gene transcription is repressed by Rev-erbα, the expression of which is induced by fibrates and which binds to a Rev-RE site adjacent to the TATA-box in the rat, but not in the human apo A-I gene promoter. The identification of rat apo A-I as a target gene for Rev-erbα suggests an implication of Rev-erbα in lipoprotein metabolism (23). Although the rat apo A-I Rev-RE is not conserved in man, Rev-erbα expression is controlled by fibrates both in rats and in man, which may point to a role for this nuclear receptor as a modulator in lipid and lipoprotein metabolism and possibly in atherosclerosis susceptibility in both species. It will be of interest, therefore, to identify target genes involved in lipid metabolism that are also under control of Rev-erbα in man.

In conclusion, our data indicate that the human Rev-erbα gene is regulated at the transcriptional level by fibrates in liver. Furthermore, this regulation is mediated by PPARα, which binds to a novel response element consisting of a 5′-A/T-rich preceded DR2 sequence. Finally, we provide evidence that PPARα and Rev-erbα bind to the same regulatory site, indicating the existence of a cross-talk between PPARα and Rev-erbα-signaling pathways.

MATERIALS AND METHODS

Cell Culture

Human hepatocytes, isolated by collagenase perfusion, and HepG2 cells were cultured exactly as described previously (12).

RNA Analysis

RNA extraction and Northern blot analysis were performed as described (3) using human Rev-erbα (43) and human acidic ribosomal phosphoprotein 36B4 (57) cDNA probes.

Construction of Recombinant Plasmids and Transfection

Cloning of the human Rev-erbα promoter fragments into pGL2 promoterless or SV40pGL2 reporter vectors (Promega, Madison, WI) and site-directed mutagenesis of Rev-erbα response elements were as described (24). Human hepatoma HepG2 cells were obtained from European Collection of Animal Cell Culture (Porton Down, Salisbury, UK). Cells were grown in DMEM, supplemented with 2 mM glutamine and 10% (vol/vol) FCS, in a 5% CO_2 humidified atmosphere at 37 C. Stimuli were dissolved in dimethylsulfoxide (DMSO). Control cells received vehicle only. All transfections were performed with a mixture of plasmids containing reporter (2 μg) and expression vectors (0.3 to 1 μg). The luciferase activity in cell extracts was determined using a luciferase assay system (Promega) following the supplier's instruction. Transfection experiments were performed in triplicate and repeated at least three times.

In Vitro Translation and EMSAs

pSG5hPPARα, pSG5mRXRα, and pSG5hRev-erbα were *in vitro* transcribed with T7 polymerase and translated using the rabbit reticulocyte lysate sytem (Promega). EMSAs with Rev-erbα, PPARα, and/or RXRα were performed exactly as described previously (2, 58). For competition experiments, increasing amounts of indicated cold probe were added just before the labeled oligonucleotide. The complexes were resolved on 5% polyacrylamide gels in 0.25× TBE buffer (90 mM Tris-borate, 2.5 mM EDTA, pH 8.3) at 4 C. Gels were dried and exposed overnight at −70 C to x-ray film (XOMAT-AR, Eastman Kodak, Rochester, NY).

Coimmunoprecipitation from Cell Extracts

HepG2 cells incubated in DMEM + 0.2% BSA were treated with fenofibric acid (0.5 mM) or vehicle (DMSO) for 24 h. Cells were subsequently washed in PBS and incubated in methionine-free DMEM supplemented with ^{35}S-labeled methionine (0.1 mCi/ml medium) for 5 h. Cells were lysed in 1 ml RIPA

buffer [20 mMTris, pH 7.5, 150 mM sodium chloride, 2 mM EDTA, 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, 0.25% (wt/vol) SDS]. Lysates were centrifuged at 100,000 × *g* for 30 min, and the supernatant was subsequently incubated with polyclonal anti-Rev-erbα antibody (S. Chopin-Delannoy and V. Laudet, manuscript in preparation) overnight at 4 C in RIPA buffer. Immune complexes were collected using protein A-Sepharose (Pharmacia, Piscataway, NJ) and washed six times in RIPA buffer. Protein complexes were separated on 10% SDS-polyacrylamide gels under reducing conditions. Gels were dried and exposed at −70 C to BIOMAX-MS film (Kodak).

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